

### REMARKS

Claims 35, 36, 38-40, 42-49, and 56 were pending in the application. Claims 35 and 40 have been amended. New claims 57-67 have been added. Accordingly, following entry of the amendments presented herein, claims 35, 36, 38-40, 42-49, and 56-67 will be pending. For the Examiner's convenience, a copy of the claims as they will be pending upon entry of the present amendment, is set forth herein as Appendix A.

No new matter has been added. Support for the amendments presented herein can be found in the specification as filed and/or the claims as previously pending. Specifically, support for the amendments to claim 35 can be found at least at page 5, lines 20-24, and page 8, lines 9-11. Support for new claim 57 can be found at least at page 39, line 15 through page 40, line 5. Support for new claim 58 can be found at least at page 1, lines 16-18 and at page 7, line 32, through page 8, line 1. Support for new claims 59-61 can be found at least at page 8, lines 29-37. Support for new claim 62 can be found at least at page 36, lines 33-34. Support for new claim 63 can be found at least at page 37, lines 19-21. Support for new claim 64 can be found at least at page 37, lines 7-8. Support for new claim 65 can be found at least at page 37, lines 8-10. Support for new claims 66 and 67 can be found at least at page 37, lines 31-38.

The foregoing claim amendments and cancellations should in no way be construed as an acquiescence to any of the Examiner's rejections, and have been made solely to expedite the prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

#### Rejection of Claims 35-36, 38-40, 42-49, and 56 Under 35 U.S.C. § 112, first paragraph

The Examiner has rejected claims 35-36, 38-40, 42-49, and 56 under 35 U.S.C. §112, first paragraph, because "the specification, while being enabling for embodiments wherein the immune response assayed is the effect of the test compound on expression of an interleukin-4 gene and wherein the maf family protein is c-Maf, does not reasonably provide enablement for practicing the claimed invention with any response and with any other maf family proteins." This rejection is respectfully traversed.

Enablement is not precluded by the necessity for some experimentation, and a considerable amount of experimentation is permitted. See In re Wands, 8 U.S.P.Q. 2d 1400, 1404 (Fed. Cir. 1988). It is Applicants' position that based on the teachings of the specification and the knowledge in the art, the ordinarily skilled artisan would be able to make and use the claimed methods without undue experimentation.

In the interest of expediting prosecution of the application, while in no way conceding to the validity of the Examiner's rejection, Applicants have amended claim 35 to recite that the maf family protein binds to a MARE regulatory sequence of a TH2-associated cytokine gene and to recite that the target DNA comprise a MARE regulatory sequence of a Th2-associated cytokine gene. The pending claims are directed to methods for identifying a compound that modulates production of a Th2-associated cytokine in a cell, comprising providing an indicator composition comprising (i) a maf family protein ***binds to a MARE regulatory sequence of a TH2 associated cytokine gene*** and (ii) a target DNA ***comprising a MARE regulatory sequence*** of a Th2-associated cytokine gene to which said maf family protein binds, wherein said indicator composition is an indicator cell or an acellular preparation; contacting the indicator composition with each member of a library of test compounds; selecting from the library of test compounds a compound of interest that modulates binding of said maf family protein to said target DNA; and determining the effect of the compound of interest on the production of a Th2-associated cytokine in a cell to thereby identify a compound that modulates production of the Th2 cytokine.

According to the amended claims, only those maf family proteins that bind to a MARE sequence of a Th2 associated cytokine gene are embraced by the claims. One of ordinary skill in the art could readily identify proteins of the maf family (e.g., as described at page 7, line 32 through page 8, line 1 of the specification) that have this activity. While the working examples of the patent focus on c-maf, other the maf family proteins were known to be structurally similar to c-maf. For example, v-maf was known in the art to be structurally similar to c-maf, having only two structural changes in the coding region from c-

maf (a substitution of an M residue at position 257 to V and fusion of the viral gag sequence to the 5' terminal end; see Kataoka *et al.* 1993. *J. Virol.* 67:2133, attached as Appendix B). The ability of a maf family protein to bind to a MARE or a Th2-associated cytokine gene could be tested using the methods as described in Example 6 or using other techniques well known in the art. Based on the teachings of the specification, one of ordinary skill in the art could readily select maf family proteins within the scope of the claims based on their ability to bind to an MARE of a Th2-associated cytokine gene.

With respect to the Th2 associated cytokine gene, the claims require that the target DNA comprise a MARE regulatory sequence of a Th2-associated cytokine gene. MARE regulatory elements and their structure are taught in the specification. For example, Applicants teach that MARE responsive elements are known in the art and include the 13 or 14 base pair elements which contain a core TRE (T-MARE) or CRE (C-MARE) palindrome (see page 8, lines 9-11). Applicants further show the existence of a MARE response element in the promoter region of IL-4 (see Example 6). Applicants note that C-maf has been shown to activate IL-10 transcription as well as IL-4 transcription (see, *e.g.*, the paper by Cao *et al.* ((2002) *J. Immunol.* 169:5715-25) attached as Appendix C.) Based on the teachings in the specification and the armed with the knowledge of one of ordinary skill in the art, the ordinary skilled artisan could readily select a target gene within the scope of the claims.

With respect to the production of TH2 cytokines, as taught in the specification, *e.g.*, at page 8 lines 31-37, and as known in the art, given the role of certain specific TH2 cytokines such as IL-4 in promoting TH2 cytokine production, an effect of a compound on *e.g.*, IL-4 or IL-10 production could be measured either directly by measuring production of IL-4 or IL-10, or indirectly by measuring production of other TH2 cytokines. One of ordinary skill in the art could readily measure Th2 cytokines using techniques described in the specification or known in the art. Exemplary techniques are taught at page 37, lines 35-39 and at pages 40, line 34 through page 41, line 4 of the specification.

Given the teachings of the specification and the knowledge of one of ordinary skill in the art, the ordinarily skilled artisan could readily perform a screening assay as claimed using a maf family protein. Exemplary assays are taught in the instant specification, e.g., at pages 36, line 4 through page 40, line 4. Accordingly, Applicants contend that the claimed invention is enabled across its breadth.

Applicants point out that the Examiner appears to doubt the utility of the claimed invention for its stated purpose, rather than the doubting the ability of one of ordinary skill in the art to practice the claimed invention. The Examiner has not set forth any evidence that would suggest that one of ordinary skill in the art would doubt the asserted utility of the claimed methods in identifying compounds that modulate production of a Th2 associated cytokine by a cell. Under 35 U.S.C. §112, first paragraph, the Examiner has the "initial burden of setting forth a reasonable explanation as to why the scope of protection provided by [the claims] is not adequately enabled by the description of the invention provided in the specification." *In re Wright*, 999 F.2d 1557 (Fed. Cir. 1993). Specifically, in *In re Brana*, 51 F.3d 1560, 1566 (Fed. Cir. 1995), it was held that:

Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility.

Additionally, the court stated that in the absence of a reason to doubt the objective truth of the teachings contained in the specification, the methods of making and using the claimed invention must be taken as complying with the requirements of §112, first paragraph. The Examiner has not met this burden, accordingly, the claims must be taken as complying with §112, first paragraph.

The Examiner also states that there is "no basis for one of skill in the art to predict the exact structural/functional characteristics of a maf protein (e.g., primary amino acid sequence of the protein and the sequence of the nucleic acid it binds) that elicits production of a Th2-associated cytokine."

As set forth above, in the interest of expediting prosecution of the application, while in no way conceding to the validity of the Examiner's rejection, Applicants have

amended claim 35 to recite that the maf family protein binds to a MARE regulatory sequence of a Th2 associated cytokine gene and to recite that the target DNA comprises a MARE regulatory sequence of a Th2-associated cytokine gene.

As set forth above, Applicants have described a genus of maf family proteins and MARE responsive elements. Applicants provide description of a sufficient variety of exemplary species to reflect the variation within the genus of maf family proteins (e.g., at page 7, line 24 through page 8, line 1 of the specification) and MARE responsive elements. Thus, structural information regarding the maf family of proteins and MARE elements to which they bind is taught in the specification and was known in the art at the time the application was filed. Thus, the instant specification satisfies the written description requirement for the claimed invention as set forth in the Written Description Guidelines (66 Fed. Reg at 1106) and by the court in *Enzo Biochem, Inc. v. Gen-Probe Inc.* (296 F.3d 1316 (Fed. Cir. 2002)). Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this section 112, first paragraph rejection of the pending claims.

Applicants further point out that claim 36 limits the maf family protein to c-maf, claim 40 limits the maf family protein to v-maf, claim 42 limits the target DNA to a sequence comprising a MARE regulatory sequence of the IL-4 gene, and claim 58 limits the maf family protein to c-maf or v-maf and the target DNA to a sequence comprising a MARE regulatory sequence of the IL-4 gene or the IL-10 gene.

In view of the foregoing, Applicants respectfully request that the Examiner reconsider and withdraw the foregoing rejection.

Rejection of Claims 35, 37-39, and 41-49 Under 35 U.S.C. § 112, second paragraph

The Examiner has rejected claims 35, 37-39, and 41-49 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. These claims are vague and indefinite and the metes and bounds of the phrase "a Maf family protein" are unclear. This rejection is respectfully traversed.

Maf family proteins were known in the art and are described in the specification as filed. The specification provides numerous examples of maf family of proteins (see e.g., page 7, line 32 through page 8, line 22), including citations for their amino acid sequences (see page 10, lines 20-35). As taught in the specification, these proteins are a subset of AP-1/CREB/ATF proteins that have homology to v-Maf, have a basic region linked to a leucine zipper domain. As taught in the specification, the small maf proteins of the maf family are taught to lack the transactivating domain. Contrary to the Examiner's position, there is an art recognized standard for identifying a Maf protein distinct from any other bZIP factor and, therefore, the metes and bounds of the term "maf family protein" are clear and definite.

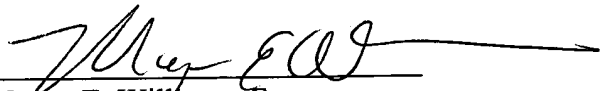
In the interest of expediting prosecution, and in no way acquiescing to the Examiner's rejection, Applicants have amended the instant claims to require that the Maf family protein bind a MARE of a Th2 associated cytokine gene, thus limiting the claim to Maf family members that have this effect. Applicants respectfully request that the Examiner reconsider and withdraw the foregoing rejection.

**CONCLUSION**

Reconsideration and allowance of all the pending claims is respectfully requested. If a telephone conversation with Applicants' Attorney would expedite prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 227-7400.

Respectfully submitted,

LAHIVE & COCKFIELD, LLP

By   
Megan E. Williams, Esq.  
Attorney for Applicants  
Reg. No. 43,270

LAHIVE & COCKFIELD, LLP  
28 State Street  
Boston, MA 02109  
Tel. (617) 227-7400

Dated: September 11, 2003

**Appendix A**  
**Pending Claims**

35. A method for identifying a compound that modulates production of a Th2-associated cytokine in a cell, comprising

providing an indicator composition comprising (i) a maf family protein which binds to a MARE regulatory sequence of a Th2 associated cytokine gene and (ii) a target DNA comprising a MARE regulatory sequence of a Th2-associated cytokine gene to which said maf family protein binds, wherein said indicator composition is an indicator cell or an acellular preparation;

contacting the indicator composition with each member of a library of test compounds;

selecting from the library of test compounds a compound of interest that modulates binding of said maf family protein to said target DNA; and

determining the effect of the compound of interest on the production of a Th2-associated cytokine in a cell to thereby identify a compound that modulates production of the Th2 cytokine.

36. The method of claim 35, wherein the maf family protein is c-Maf.

38. The method of claim 35, wherein the Th2-associated cytokine gene is an interleukin-4 gene.

39. The method of claim 35, wherein the effect of the compound of interest on Th2-associated cytokine production is determined by determining the effect of the compound on development of T helper type 1 (Th1) or T helper type (Th2) cells.

40. The method of claim 35, wherein the maf family protein is v-maf.

42. The method of claim 35, wherein the target DNA comprises the regulatory sequence of an interleukin-4 gene.

44. The method of claim 35, wherein the indicator composition is an indicator cell.



44. The method of claim 43, wherein the indicator cell is a lymphoid cell.
45. The method of claim 44, wherein the lymphoid cell is a Th2 cell.
46. The method of claim 44, wherein the lymphoid cell is a Th1 cell.
47. The method of claim 44, wherein the lymphoid cell is a B cell.
48. The method of claim 43, wherein the indicator cell is a non-lymphoid mammalian cell.
49. The method of claim 43, wherein the indicator cell is a yeast cell.
56. The method of claim 44, wherein lymphoid cell is a helper precursor (Thp) cell.
57. The method of claim 35, wherein the indicator composition is an acellular preparation.
58. The method of claim 35, wherein the maf family protein is c-maf or v-maf and the Th2-associated cytokine gene is an IL-4 gene or an IL-10 gene.
59. The method of claim 35, wherein the Th2-associated cytokine gene is an interleukin-10 gene.
60. The method of claim 35, wherein the production of IL-4 is modulated.
61. The method of claim 35, wherein the production of IL-10 is modulated.
62. The method of claim 35, wherein the maf family protein is recombinantly expressed in a cell.
63. The method of claim 35, wherein the cell does not naturally express the maf family protein.

64. The method of claim 35, wherein the regulatory sequence comprises about 3 kb of the upstream regulatory sequences from the IL-4 promoter.

65. The method of claim 35, wherein the regulatory sequence comprises nucleotides -157 to +58 of the IL-4 promoter.

66. The method of claim 35, wherein Th2-associated cytokine production can be assessed by detecting cytokine mRNA.

67. The method of claim 35, wherein Th2-associated cytokine production can be assessed by detecting the cytokine protein.

JOURNAL OF VIROLOGY, Apr. 1993, p. 2133-2141  
0022-538X/93/042133-09\$02.00/0  
Copyright © 1993, American Society for Microbiology

Vol. 67, No. 4

## Structure-Function Analysis of the *maf* Oncogene Product, a Member of the b-Zip Protein Family†

KOHSUKE KATAOKA,<sup>1,2</sup> MAKOTO NISHIZAWA,<sup>1\*</sup> AND SADAANKI KAWAI<sup>2</sup>

Department of Viral Oncology, Cancer Institute, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170,<sup>1</sup>  
and Department of Tumor Virus Research, Institute of Medical Science, University of Tokyo,  
4-6-1 Shirokanedai, Minato-ku, Tokyo 108,<sup>2</sup> Japan

Received 31 August 1992/Accepted 28 December 1992

The *v-maf* oncogene, identified as the transforming gene of the avian retrovirus AS42, encodes a protein containing a b-Zip motif. From this structural feature, the v-Maf protein was expected to form a dimer and function as a nuclear DNA-binding protein. In this study, we demonstrate that this protein indeed localizes predominantly in the nucleus and forms a homodimer through its leucine zipper structure. To delineate the structural requirement for the transforming activity, we constructed and characterized a panel of *v-maf* mutants harboring various deletions or point mutations. A region of about 100 amino acid residues located near its carboxyl terminus, which contains the b-Zip motif, was found to be essential for the basal transforming activity of v-Maf on chicken embryo fibroblasts. On the other hand, the amino-terminal two-thirds of the v-Maf protein seems to play a role in potentiating the transforming activity of v-Maf. It was also found that the *c-maf* proto-oncogene, without any structural modification in its protein-coding region, could transform cells as efficiently as could the *v-maf* oncogene when transduced by a retroviral vector. Thus, it is probably deregulated expression that makes the *v-maf* gene oncogenic. In addition, we discovered one point mutation, altering the structure of the b-Zip domain, which further enhances the transforming activity of the *v-maf* oncogene. Such mutant will be useful in exploring the mechanism of action of the Maf protein.

We recently isolated an avian transforming retrovirus, AS42, from a spontaneous musculoaponeurotic fibrosarcoma of chicken. This virus when inoculated into newborn chickens induces tumors which are pathologically indistinguishable from the original tumor. It also induces transformation of chicken embryo fibroblast (CEF) cells in vitro. The unique pathogenicity of the AS42 virus prompted us to analyze the structure of its proviral DNA, which led to the identification of a new oncogene, *maf* (musculoaponeurotic fibrosarcoma) (29). The virus is replication defective, and the only viral product is the Gag-Maf fusion protein of about 100 kDa (12). The most striking structural feature of the v-Maf protein is the long repeats of glycines and histidines in its middle portion. Interestingly, variant AS42 viruses with small deletions, apparently generated by homologous recombination among the GGC repeats encoding the glycine stretches, accumulate in the stocks as we passage this virus (12).

The v-Maf protein contains another characteristic structure, the b-Zip motif, in its carboxyl terminus. This motif has been proposed to serve as a dimerizing and DNA binding domain in several transcriptional regulators, including Fos and Jun (20, 36). Heptad repeats of leucine residues in highly alpha-helix-permissive sequence, the leucine zipper structure, are believed to play an important role in dimerization of the proteins by coiled-coil-type interaction. The leucine zipper structures are usually preceded by a positively charged region of about 30 amino acid residues, proposed to serve as a DNA binding domain. The putative DNA binding domain of Maf shares significant (20 to 30%) homology with those of the other known leucine zipper proteins (29).

The *fos* and *jun* oncogene products are the most extensively analyzed b-Zip proteins. The c-Fos/c-Jun heterodimer and the c-Jun homodimer are the major components of the transcription factor AP-1, which binds selectively to the phorbol 12-*O*-tetradecanoate-13-acetate (TPA)-responsive element of several vertebrate genes (3, 5, 8, 30). Furthermore, Jun also forms heterodimers with some CREB proteins through the leucine repeat motif and binds to the cyclic AMP (cAMP)-responsive element (1, 10, 23). In these protein complexes, correct arrangement of the DNA binding domains seem to be brought about by dimerization, because the ability to dimerize is a prerequisite for the DNA-binding property of these proteins.

The presence of a typical b-Zip motif strongly suggests that Maf is a nuclear protein which forms dimers and acts as a transcriptional regulator. In this study, we examined this hypothesis by using a retroviral expression system in CEF cells. Nuclear localization and the dimer-forming ability of Maf protein were confirmed, and the transforming ability of the *c-maf* proto-oncogene transduced by the retroviral vector was demonstrated. Furthermore, analysis of a series of *v-maf* mutants revealed that the regions of v-Maf essential for dimer formation were also essential for the transforming activity. The results are consistent with the model that v-Maf protein functions as a dimer in the nucleus to exert its transforming activity.

### MATERIALS AND METHODS

**Cells and viruses.** Preparation of CEF cells, focus-forming assay of viruses, and soft-agar colony formation were carried out as described previously (13, 16, 37). DNA transfection was performed by the polycation-dimethyl sulfoxide method described previously (14). We recovered the recombinant viruses which encode the mutated *maf* gene as described by Semba et al. (32) and assayed its transforming

\* Corresponding author.

† Dedicated to the memory of Sadaaki Kawai, who died on 3 August 1990.

activity by colony formation in soft-agar suspension culture. When no transforming activity was detected in the supernatant of the transfectant, virus production was confirmed by Western immunoblot analyses of the infected cell lysates, using the anti-Maf serum and/or an antiserum against the viral Gag protein.

**Preparation of Maf-specific antibodies and immunological experiments.** A mutated v-Maf protein which has a deletion of five amino acid residues in the putative DNA binding domain was bacterially expressed as follows. The *NcoI*-*MluI* fragment of a deletion mutant of the *v-maf* gene, *Md26.22*, was excised from a derivative of plasmid pRAM, *pMd26.22* (see below), and was treated with Klenow fragment; this procedure was followed by addition of an *XbaI* linker and *XbaI* digestion. The resultant *XbaI* fragment was introduced into the *NheI* site of the pET-3a vector, an *Escherichia coli* expression vector developed by Rosenberg et al. (31). From this construct, the mutant v-Maf protein, MD26.22, was expressed as a fusion protein in which the amino-terminal four amino acid residues should be derived from the vector and the *XbaI* linker sequences. *E. coli* cells transformed with this plasmid were cultured in rich medium and were induced to produce the recombinant protein in early log phase by addition of isopropyl- $\beta$ -D-thiogalactopyranoside. We found that the recombinant protein was accumulated as an inclusion body in bacterial cells. We partially purified the accumulated protein to more than 90% homogeneity by washing the inclusion body fraction with Triton X-100 and EDTA as described previously (24). The recombinant protein was further purified on a preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel and was used as an antigen to immunize animals. Two mouse hybridoma clones which produce monoclonal antibodies against the v-Maf protein were established by the general method (11). By preliminary epitope mapping experiments, it was shown that two monoclonal antibodies, MovN1 and MovC1, recognize the amino- and carboxyl-terminal portions, respectively, of the v-Maf protein.

For indirect immunofluorescent staining, virus-infected or uninfected CEF cells were fixed with cold methanol. v-Maf-specific rabbit antiserum and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G serum were diluted with phosphate-buffered saline (PBS) and were used as primary and secondary antibodies, respectively. Heat-denatured cell lysate of the virus-infected cells were prepared as described previously (7). Immunoprecipitation experiments were performed as described previously (15).

**Retroviral expression vector system and construction of v-maf mutants.** The amino acid and nucleotide residues were numbered from the 5' recombination point between the viral *gag* gene and *v-maf* gene as previously described (29). To generate a Rous sarcoma virus variant which carries the *v-maf* gene instead of the *v-src* gene, the *NcoI*-*Bsu36I* fragment (nucleotides 53 to 1123) was excised from the provirus clone of AS42 and was ligated to the 34-bp *BalI*-*NcoI* fragment derived from the region just upstream of the translational initiator ATG codon of the *v-src* gene of Rous sarcoma virus, resulting in good agreement with the consensus sequence around the initiator methionine residue (18). The *BalI*-*Bsu36I* fragment was then treated with Klenow fragment, attached to an *MluI* linker, and subjected to *MluI* digestion. The *MluI* fragment was recloned into pUC-9(*MluI*), a derivative of pUC-9, which was constructed by addition of an *MluI* linker between the *HincII* site and *SmaI* site of plasmid pUC-9. We named this plasmid pRAM. This construct and pRAM(-), in which the direction of the insert

is opposite that of pRAM, were used for in vitro mutagenesis. To test its transforming activity, we excised the insert of pRAM by *MluI* digestion and recloned it into the *MluI* site of the pRV-2 retroviral vector plasmid (32), after which CEF cells were transfected. Deletion mutants and point mutants of the *v-maf* gene were constructed as follows (also see Table 1 and Fig. 5).

To construct *pNd1*, *pNd2*, *pNd3*, and *pNd4*, pRAM(-) plasmid DNA was digested by *NcoI* and was gradually deleted by exonuclease BAL 31 from its termini; this procedure was followed by treatment with Klenow fragment and addition of an *NcoI* linker (pCCCATGGG). We then excised the *NcoI*-*HindIII* fragments of various lengths and replaced the *NcoI*-*HindIII* fragment of pRAM(-) with these fragments to generate deletion mutants of pRAM(-). By sequencing analyses of the resultant clones, we chose four in-frame deletion constructs of various lengths and named them *pNd1* to -4. In these amino-terminal deletion mutants, the putative initiator ATG codons were donated by the *NcoI* linker, and the 5' preceding sequences were provided by the pRAM(-) plasmid to generate good agreement with Kozak's consensus sequence (18).

To make *pNd5*, the *NcoI*-*HaeIII* fragment (nucleotides 53 to 719) of pRAM was deleted and replaced by an *NcoI* linker.

To make *pNd6*, pRAM was digested with *NcoI* and *BstEII*, blunt ended, and then self-ligated.

To make *pCd1*, *pCd2*, *pCd3*, and *pCd4*, deletions from the carboxyl terminus were introduced by insertion of an *NheI* linker (pCTAGCTAGCTAG), which enters terminator codons in all three frames, into the following restriction sites: *pCd1*, *BamHI* (nucleotide 1036); *pCd2*, *RsaI* (nucleotide 999); *pCd3*, *BglII* (nucleotide 956); and *pCd4*, *BstEII* (nucleotide 756).

*pNd5Cd2* was generated from *pNd5* and *pCd2*.

To make *pVd1*, *pVd3*, and *pVd7*, the *BssHII*-*BstEII* fragment (nucleotides 509 to 756) of pRAM was replaced by the corresponding fragments of size variants of AS42 virus clones (12) B1, B3, and B7, respectively.

*pMd23* and *pMd24* were obtained by deleting *BssHII*-*BssHII* (nucleotides 509 to 599) and *BssHII*-*BstEII* (nucleotides 509 to 756) fragments from pRAM, respectively.

To make *pMd23.5*, *pMd24* was digested with *BstEII*, treated with Klenow fragment, and digested with *EcoRI*. The resulting 3.2-kb fragment, which contains pUC vector sequence, was excised and was ligated to the 0.4-kb *HaeIII* (nucleotide 718)-*EcoRI* fragment of pRAM to generate *pMd23.5*.

Deletion mutants *pMd45*, *pMd56*, *pMd15*, *pMd16*, and *pMd46* were generated by partial digestion with *PvuII* and self-ligation.

To make *pMd26.22*, the *Ksp632I*-*EcoO109I* fragment (nucleotides 808 to 838) of pRAM was replaced by the following synthetic oligonucleotide, resulting in deletion of five amino acid residues (amino acids 276 to 280):

5'-CAAGGAAGAGGTGATCCGGCT-3'  
3'-CCTTCTCCACTAGGCCGACTG

*pVp* was constructed by exchanging the *BstEII*-*BamHI* fragment (nucleotides 756 to 1036) of pRAM by the corresponding fragment of AS42 provirus clone B1. Other point mutants of the *v-maf* gene were obtained by the method of Kunkel et al. (19). A construct that expresses the c-maf product was produced by insertion of synthetic nucleotide sequence shown below into *NcoI* site of *pVp*:

5'-CATGGCATTAGAGCTCGCAATGAGCGGGCTCCGACCTGCCACCAAGTCCCTGGC-3'  
 3'-CGTAGTCTCGAGCGTTACTCGCCGAGGCTGGACGGGTGGTCAGGGGACCGGTAC-5'

In this report, mutated genes of *maf* are indicated by italics (e.g., *Md26.22*), and their products are in capital letters (e.g., MD26.22).

**In vitro transcription and translation.** Each mutant of the *v-maf* gene was recloned into plasmid pGEM-3 or pGEM-4 for in vitro transcription. The plasmid DNAs were linearized and were used as templates for T7 RNA polymerase. In vitro translation was performed in the presence of [<sup>35</sup>S]methionine, using wheat germ extract purchased from Promega Corp. (Madison, Wis.). Cross-linking experiments were performed as follows. In vitro-translated proteins were dialyzed against PBS and were reacted with glutaraldehyde (final concentration, 0.005%) at room temperature for 30 min. The reaction was stopped by addition of 1/10 volume of 1 M Tris hydrochloride. The cross-linked products were concentrated by acetone precipitation and were separated by SDS-polyacrylamide gel electrophoresis.

## RESULTS

**Construction of replication-competent retrovirus that expresses the *v-maf* oncogene.** To test the biological activity of the *v-maf* oncogene, we used a replication-competent retrovirus vector, pRV-2 (32). First, the *v-maf* sequence excised from the AS42 proviral DNA was subcloned to produce plasmid pRAM. The insert of this plasmid lacks all of the viral *gag* sequence and the 5'-terminal 54-bp portion of *v-maf* (Fig. 1 and Materials and Methods). We named the amino-terminal truncated form of the *v-maf* gene in plasmid pRAM *Pt*. Subsequently, the insert of this plasmid was recloned into the unique *Mlu*I site of pRV-2 to produce pRV-2/*Pt*. The plasmid was transfected into CEF cells, and a replication-competent recombinant virus, harboring the truncated *v-maf* gene, was recovered. This virus showed transforming activity when infected into fresh CEF cells. The frequency and morphology of the foci (Fig. 2) and colonies induced by this virus were comparable to those induced by the AS42 virus, indicating that the Gag portion and the amino-terminal 18 amino acids of v-Maf are dispensable for its transforming activity. In the following experiments, we used plasmid pRAM as a substrate for in vitro mutagenesis and to assay the transforming potential of the mutated genes, using the retroviral vector system.

The *v-maf* gene product localizes predominantly in the nucleus. To prepare antibodies against v-Maf, we tried to produce the v-Maf protein in bacterial cells. However, all attempts to express the intact *v-maf* gene by using several expression vector systems were unsuccessful, and it was therefore apparent that the v-Maf protein was toxic to bacterial cells. We then tried to express a deletion mutant of *v-maf* gene, *Md26.22*, in *E. coli* cells, using the pET3-a expression vector (31). The product of *Md26.22* lacks five amino acid residues in its putative DNA binding domain and was expected to be biologically inactive (see below). Upon induction of the *E. coli* cells transformed with this plasmid, a protein of about 40 kDa was produced (data not shown). We partially purified the MD26.22 protein and used it for immunizing the animals. The resulting serum could efficiently precipitate the 100-kDa Gag-Maf fusion protein from the lysate of AS42-infected CEF cells (Fig. 3, lane 2). The immunoprecipitation band pattern was unchanged before and after heat denaturation of the lysate (compare lanes 2

and 5), suggesting that no other proteins were associated under these conditions. A protein of about 42 kDa was also specifically immunoprecipitated from the lysate of the cells infected with the v-Maf-containing replication-competent virus RV-2/*Pt*, described above (lane 8). In contrast to the AS42-infected cells, RV-2/*Pt* induced additional minor bands of around 80 kDa. The origin of these minor bands is not clear, but they may have resulted from recombinational events in the virus genome and do not seem to be Maf-associated proteins because these bands were not abolished by heat denaturation of the cell lysates prior to immunoprecipitation (lane 11). The results indicate that this serum recognizes the v-Maf protein with reasonably good specificity.

To examine the subcellular distribution of the v-Maf protein, we performed indirect immunofluorescence staining of the virus-infected cells, using the anti-v-Maf antiserum. Strong staining was observed within the nuclei of the AS42-infected and RV-2/*Pt*-infected CEF cells (Fig. 4A and C). No significant fluorescence was observed when the virus-infected cells were stained with preimmune serum (Fig. 4B and D) or when uninfected CEF cells were stained with the anti-Maf serum (data not shown). These results demonstrate the nuclear localization of the v-Maf protein.

**Dimer-forming abilities of the v-Maf protein.** The b-Zip motif of the v-Maf protein suggests that this protein may form homodimers or associate with other protein(s). Therefore, we first tested the abilities of the v-Maf proteins to form

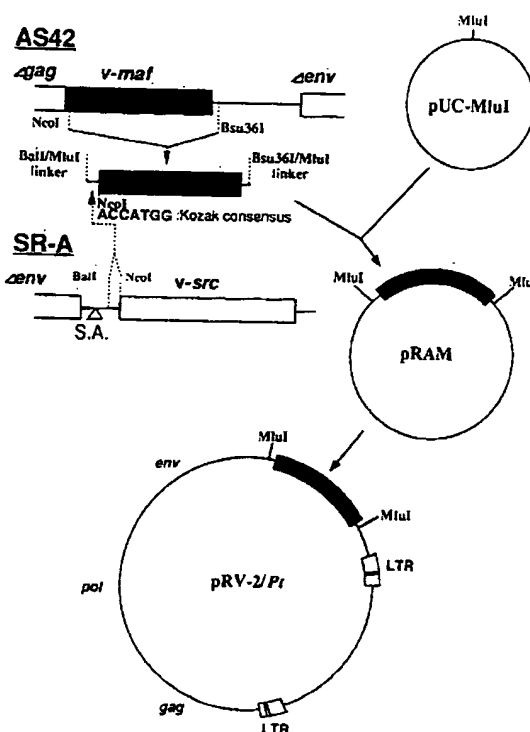


FIG. 1. Construction of replication-competent retrovirus which harbors the *v-maf* oncogene. Details of construction are given in Materials and Methods. S.A., splice acceptor; LTR, long terminal repeat.

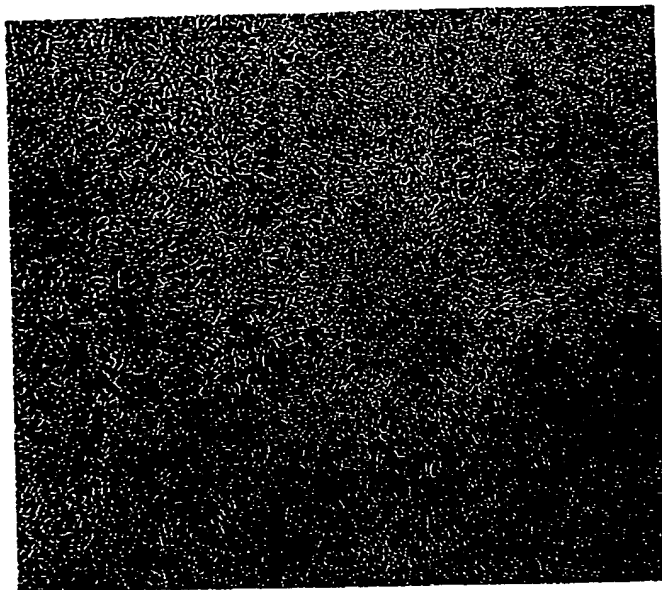


FIG. 2. A focus of transformed cells induced by the RV-2/Pt virus. CEF cells were infected with the RV-2/Pt virus, overlaid with 0.7% agar medium, and incubated at 37°C for 1 week.

dimers by cross-linking experiments. For this purpose, we produced two v-Maf mutants, ND1 and ND4 (ND stands for N-terminal deletion), with amino-terminal deletions of different sizes by transcription and translation *in vitro* (Fig. 5A). The products were cross-linked with glutaraldehyde and subjected to SDS-polyacrylamide gel electrophoresis. Bands of about twice the molecular weight of the mutant products which probably represent homodimers appeared after cross-linking of each protein (Fig. 6, lane 1 to 4). When the two mutant proteins were cotranslated and cross-linked, an additional band of intermediate size appeared, suggesting the formation of a dimer between the two size variants of the v-Maf protein (lane 5). In contrast, when we used another v-Maf mutant, ND1-L2PL4P, in which the helix-permissive

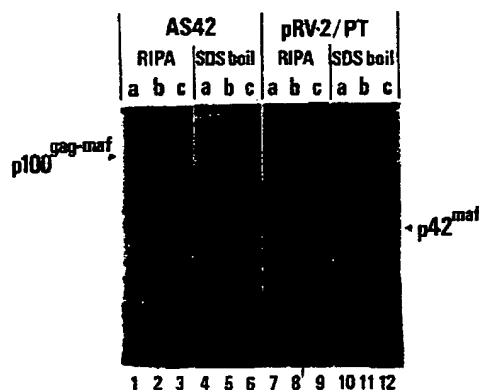


FIG. 3. Immunoprecipitation of v-maf gene products. AS42-infected (lane 1 to 6) or RV-2/Pt virus-infected (lane 7 to 12) transformed CEF cells were labeled with [<sup>35</sup>S]methionine. Non-denatured (lanes 1 to 3 and 7 to 9) or heat-denatured (lanes 4 to 6 and 10 to 12) extracts of the labeled cells were immunoprecipitated with normal mouse serum (a; lanes 1, 4, 7, and 10), mouse anti-Maf serum (b; lanes 2, 5, 8, and 11), and mouse anti-Maf serum preincubated with the v-Maf antigen produced in *E. coli* (c; lanes 3, 6, 9, and 12).

structure was destroyed by substitutions of leucine residues by proline residues, no cross-linked products were detected (lanes 6 and 7). This result indicates that dimer formation is dependent on the leucine zipper motif of the v-Maf protein.

To further confirm dimerization of the Maf protein, we performed coimmunoprecipitation experiments using two mouse monoclonal antibodies, MovN1 and MovC1, which recognize the amino and carboxyl termini, respectively, of the v-Maf protein. The PT protein, the nearly full-length v-Maf protein produced from plasmid pRAM, was efficiently precipitated by each of the two antibodies (Fig. 7, lane 1 and 2), whereas the L2PL4P protein, which carries substitution of two leucine residues, could not be recognized by the MovC1 antibody (lane 3). When the amino-terminal deletion mutant ND4, which itself cannot be recognized by the N-terminal-specific MovN1 antibody, was cotranslated with PT and immunoprecipitated with this antibody, ND4 was coprecipitated (compare lanes 6 and 7), indicating complex formation between the two forms of the v-Maf protein. However, when the L2PL4P mutant protein instead of the PT protein was coexpressed with the ND4 protein, ND4 was not coprecipitated by MovN1 (lane 8), again indicating that complexing is dependent on the leucine repeat.

To examine additional structures required for homodimer formation, we constructed a panel of deletion mutants and point mutants of the v-maf gene and examined the dimer-forming abilities of their products. In parallel, we also tested transforming abilities of the mutated genes by using the avian retrovirus vector system (see below). All of the v-maf mutants were constructed from plasmid pRAM or its derivative, and their structures are summarized in Fig. 5 and Table 1 (also see Materials and Methods). In addition to the amino-terminal deletion mutants *Nd1* to *Nd6*, we constructed carboxy-terminal deletion mutants *Cd1* to *Cd4* (*Cd* stands for C-terminal deletion). Three internal deletion mutants (*Vd1*, *Vd3*, and *Vd7*) of v-maf were constructed by using the proviral DNA of the naturally occurring size variants of AS42 virus. In these clones, internal 96 to 150-bp sequences have been deleted by homologous recombination between the three GGC repeats which correspond to the glycine tracts (12).

The dimer-forming abilities of the mutant proteins were demonstrated by chemical cross-linking experiments. *In vitro* translation of the PT form of the Maf protein gave rise to multiple bands which could be derived as a result of initiation from internal methionine codon, premature termination, or posttranslational modifications (Fig. 8, lane 1). Therefore, for some of the mutants, double-mutant proteins (ND2CD1, ND2CD2, ND2CD3, ND2MD56, ND2L2P, and ND2L2PL4P) made in the background of an amino-terminal deletion mutant (ND2) were used instead of full-length proteins to obtain clearer results. As shown in Fig. 8, consistent with the leucine zipper model, when the heptad repeats of the leucine residues remained intact (although the fifth is substituted by a tyrosine residue in Maf), the Maf proteins (PT, ND2, and ND2CD1) efficiently formed dimers (Fig. 8, lane 1 to 6). When the sixth leucine residue was deleted (ND2CD2), decreased dimer formation was observed (lane 8). Furthermore, if the carboxy-terminal three leucine repeats were deleted (ND2CD3), dimer formation was no longer detected (lane 10). The ND2L2P protein, which has substitution of the second leucine residue by proline, formed homodimers as efficiently as did the wild-type protein (lane 14), but an additional point mutation of the fourth leucine residue to proline (ND2L2PL4P) completely abolished the dimer-forming ability of v-Maf (lane 16). In

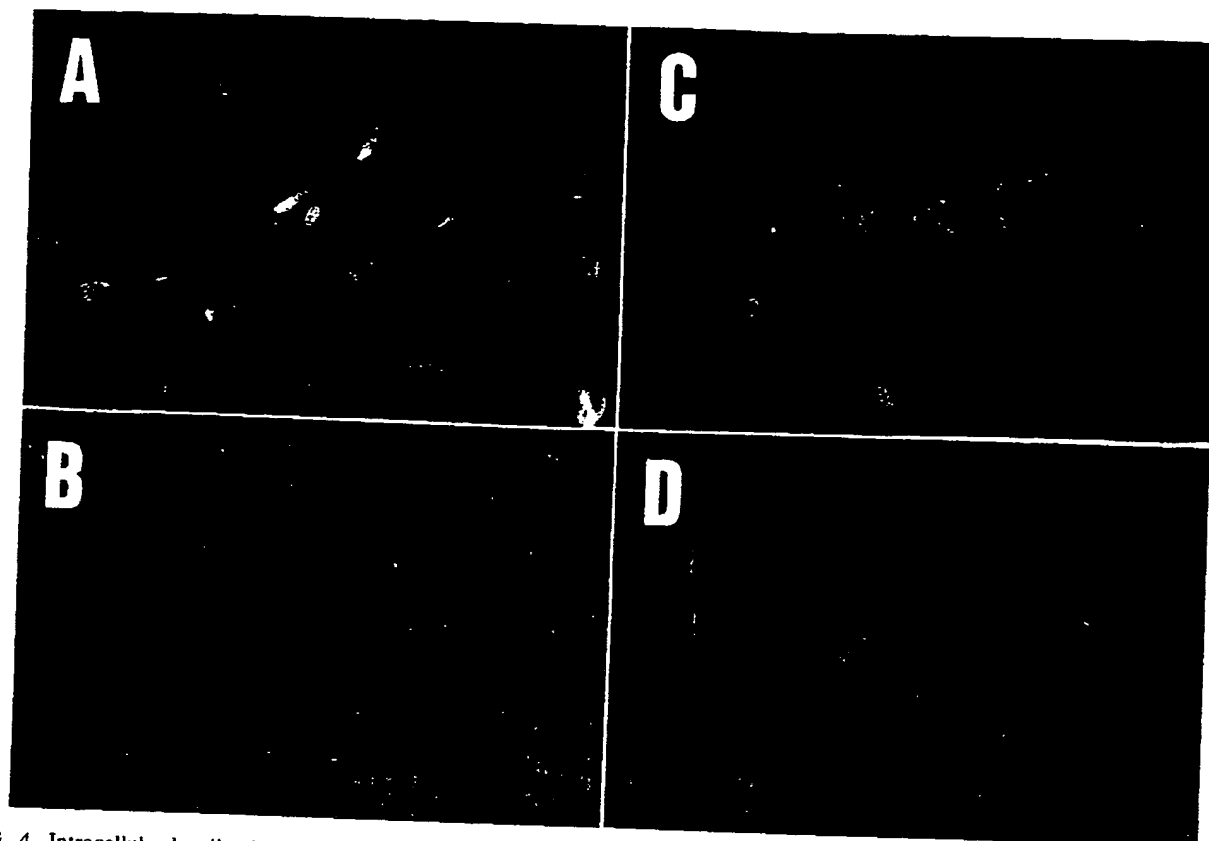


FIG. 4. Intracellular localization of v-Maf proteins in CEF cells by indirect immunofluorescence. CEF cells infected with the AS42 or RV-2/Pt virus were stained with anti-Maf rabbit serum or with normal serum. Fluorescein isothiocyanate-conjugated antibodies to rabbit immunoglobulin were used as the second antibody. (A) AS42 virus-infected CEF cells stained with anti-Maf serum; (B) AS42-infected cells reacted with preimmune rabbit serum in place of the anti-Maf serum; (C) RV-2/Pt virus-infected CEF cells stained with anti-Maf serum; (D) RV-2/Pt virus-infected cells treated with preimmune serum.

contrast, as summarized in Fig. 5, point mutations or a small deletion in the putative DNA binding domain showed no effects on dimer-forming abilities (data not shown). These results clearly demonstrate that the leucine zipper structure is responsible for the dimer-forming ability of the v-Maf protein.

**Identification of the v-maf region essential for its transforming activity.** Transforming activities of the mutant *maf* genes were examined by using the retroviral vector system described above; the results are summarized in Fig. 5.

Among the amino-terminal deletion mutants, *Nd1* to *Nd4* could induce CEF cells to form colonies in soft agar, although the number and size of the transformed colonies gradually decreased as the deletions got longer (data not shown). The two shorter mutants, *Nd5* and *Nd6*, were no longer transforming. On the other hand, the VD7 protein (lacking two of the three glycine stretches) and the MD23 protein (lacking the histidine stretch and the first glycine stretch) both retained colony-inducing activity. Even the MD23.5 protein (lacking all of the four single-amino-acid stretches) retained some transforming activity. These results indicate that the amino-terminal two-thirds of the v-Maf protein is not essential for its basal transforming activity but is required for full activity.

The transforming activities of the carboxy-terminal deletion mutants showed good correlation with their dimer-forming abilities. The CD1 and CD2 mutants, which retained dimer-forming ability, also retained transforming activity,

while the larger deletions of the CD3 and CD4 proteins destroyed both dimer-forming ability and transforming activity.

From the deletion experiments, a region of about 100 amino acid residues (indicated by the arrow at the bottom of Fig. 5A) was found to be essential for the transforming activity of v-Maf. This minimum essential region includes almost the entire b-Zip motif.

As a complementary experiment, we tested the transforming activity of the v-Maf gene harboring point mutations or a small deletion in the minimal essential region (Fig. 5B). Substitutions of one or two leucine residues of the leucine zipper motif with prolines abolished the transforming activity (L2P and L2PL4P). On the other hand, substitution of the basic amino acid residues located in the putative DNA binding domain with acidic amino acids (R10D, K19E, R22E, and KRR.EEE) and introduction of a small deletion into the same region (MD26.22) all resulted in the loss of transforming activity. Likewise, substitution of a conserved alanine residue in the same region with other amino acids (A14V and A14D) also abolished the transforming activity. In contrast, another point mutant, Q5H, in which a glutamine residue well conserved among b-Zip proteins was substituted with histidine, unexpectedly showed increased transforming potential. The recombinant virus expressing the Q5H mutant protein was found to induce denser and clearer colonies at about 20-fold-higher efficiency than did the RV-2/Pt virus (Fig. 9).

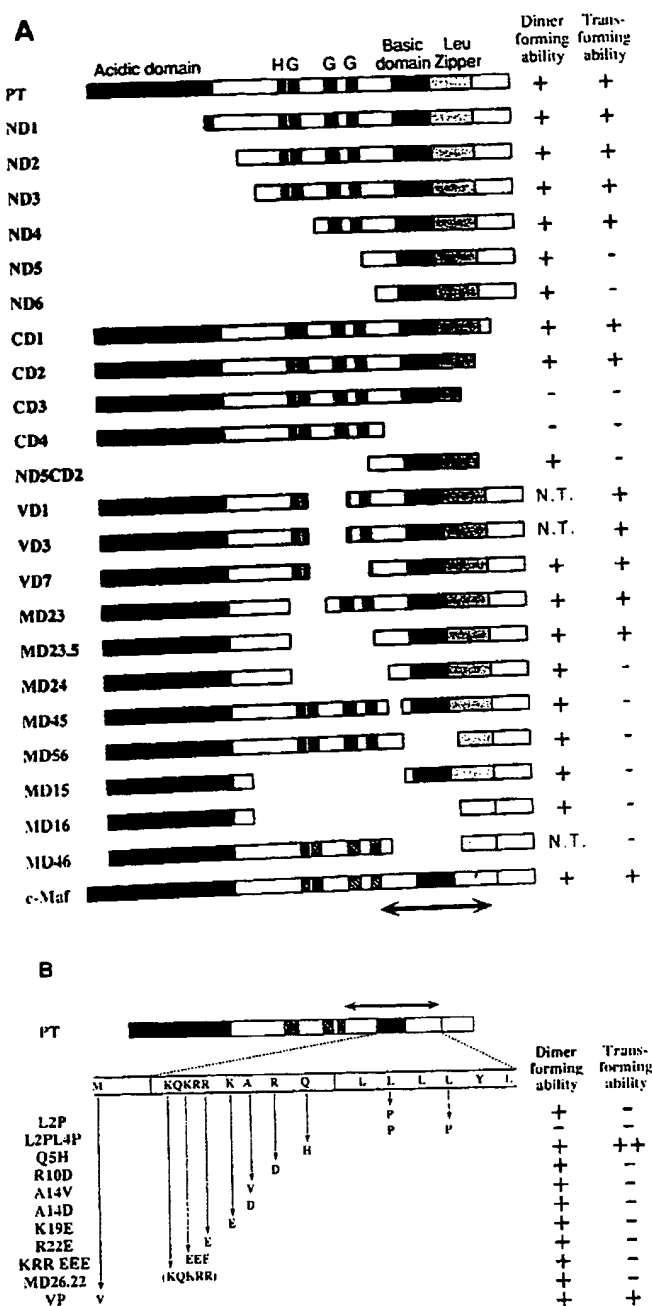


FIG. 5. Schematic representation of v-Maf mutant proteins constructed in this study and their transforming and dimer-forming abilities. (A) Deletion mutants; (B) small deletion and substitution mutations introduced into the minimal essential domain for its transforming ability. Arrows at the bottom of panel A and top of panel B indicate the minimal essential region required for colony-inducing activity of v-Maf.

**Overexpression of the c-Maf protein is sufficient for transformation of CEF cells.** Analyses of the genomic and cDNA clones of the *c-maf* gene indicate that the methionine codon located immediately downstream of the junctional site between the viral *gag* sequence and the *v-maf* sequence in AS42 virus coincides with the initiation codon of the c-Maf protein (unpublished data). In addition to the fusion of the

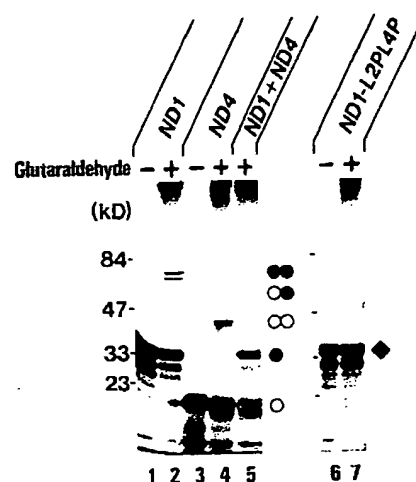


FIG. 6. Demonstration of homodimer formation of the v-Maf protein. In vitro-translated proteins were cross-linked by glutaraldehyde treatment and were resolved by SDS-polyacrylamide gel electrophoresis. The positions of the two amino-terminal deletion mutant gene products of different lengths, ND1 and ND4, and their dimers are indicated by closed and open circles, respectively. The diamond indicates the position of a mutant of Maf, ND1-L2PL4P. Numbers on the left indicate the molecular sizes of marker proteins.

viral Gag-Maf fusion, the AS42 v-Maf protein deduced from the nucleotide sequence of a cloned provirus was found to have another structural difference compared with the c-Maf protein: a valine-to-methionine substitution at position 257. However, recently analyzed size variants of AS42 virus do not contain this base substitution, suggesting that the previously analyzed clone, from which all of the v-Maf constructs used in this study were derived, represents a rare point mutant of AS42 (12). We constructed the pVp clone, which encodes a Maf protein containing a valine at position 257, and found that this construct shows no clear alteration in either dimer-forming ability or transforming activity. Furthermore, we reintroduced the deleted amino-terminal 54 nucleotides back into the pVp construct and found that the

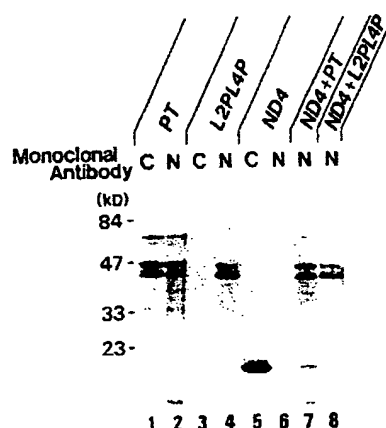


FIG. 7. Demonstration of dimer formation of Maf protein by coimmunoprecipitation. Either of two mouse monoclonal antibodies, MovN1 (N; lanes 2, 4, and 6 to 8) or MovC1 (C; lanes 1, 3, and 5), was used to immunoprecipitate <sup>35</sup>S-labeled in vitro-translated v-Maf proteins (indicated at the top). Numbers on the left indicate the molecular sizes of marker proteins.



Vol. 67, 1993

TRANSFORMING ACTIVITY OF Maf

2139

TABLE 1. Amino acid compositions of Maf mutants

Mutant	Amino acids present <sup>a</sup>	Total amino acids
PT	<sup>19</sup> M to <sup>369</sup> M	351
ND1	<u>MG</u> <sup>114</sup> V to <sup>369</sup> M	258
ND2	<u>MG</u> <sup>139</sup> A to <sup>369</sup> M	233
ND3	<u>MG</u> <sup>154</sup> A to <sup>369</sup> M	218
ND4	<u>MG</u> <sup>203</sup> P to <sup>369</sup> M	169
ND5	<u>M</u> <sup>240</sup> G to <sup>369</sup> M	131
ND6	<u>M</u> <sup>253</sup> V to <sup>369</sup> M	118
CD1	<sup>19</sup> M to <sup>347</sup> S	329
CD2	<sup>19</sup> M to <sup>333</sup> KSS	317
CD3	<sup>19</sup> M to <sup>320</sup> ILAS	317
CD4	<sup>19</sup> M to <sup>254</sup> T	305
ND5CD2	<u>M</u> <sup>240</sup> G to <sup>333</sup> KSS	236
VD1	<sup>19</sup> M to <sup>188</sup> G and <sup>222</sup> G to <sup>369</sup> M	97
VD3	<sup>19</sup> M to <sup>188</sup> G and <sup>221</sup> G to <sup>369</sup> M	318
VD7	<sup>19</sup> M to <sup>188</sup> G and <sup>239</sup> G to <sup>369</sup> M	319
MD23	<sup>19</sup> M to <sup>171</sup> A and <sup>202</sup> P to <sup>369</sup> M	301
MD23.5	<sup>19</sup> M to <sup>171</sup> AVT and <sup>241</sup> L to <sup>369</sup> M	321
MD24	<sup>19</sup> M to <sup>171</sup> A and <sup>253</sup> V to <sup>369</sup> M	284
MD45	<sup>19</sup> M to <sup>251</sup> Q and <sup>264</sup> L to <sup>369</sup> M	270
MD56	<sup>19</sup> M to <sup>263</sup> Q and <sup>309</sup> L to <sup>369</sup> M	339
MD15	<sup>19</sup> M to <sup>137</sup> Q and <sup>264</sup> L to <sup>369</sup> M	306
MD16	<sup>19</sup> M to <sup>137</sup> Q and <sup>309</sup> L to <sup>369</sup> M	225
MD46	<sup>19</sup> M to <sup>251</sup> Q and <sup>309</sup> L to <sup>369</sup> M	180
c-Maf	<sup>19</sup> M to <sup>369</sup> M	294
		369

<sup>a</sup> Codon numbers from the gag-maf fusion point are indicated to the left of amino acids. Amino acid residues added in the course of construction are underlined.

resulting c-Maf protein forms a homodimer and induces transformation of CEF cells as efficiently as does the PT protein (data not shown).

### DISCUSSION

In this study, we have demonstrated that, as expected from its structure, the v-Maf protein localizes to the nucleus and forms homodimers through its leucine zipper motif. In addition, we constructed a series of deletion mutants of the v-maf gene to determine the minimum essential region for its transforming activity and found that the amino-terminal

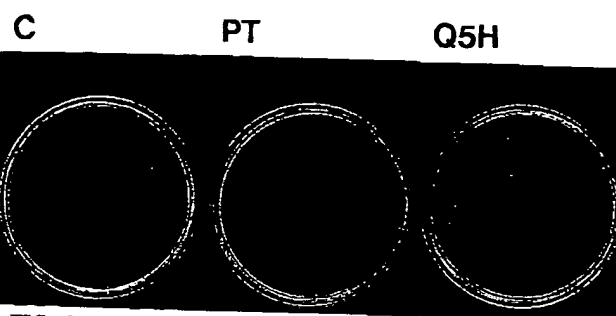


FIG. 9. Enhanced colony-inducing activity of the Q5H mutant. C, uninfected normal CEF cell culture; PT, RV-2/Pr virus infected cell; Q5H, cell culture infected with RV-2/Q5h virus.

two-thirds of the v-Maf protein was not essential for basal transforming ability but has an enhancing effect on transforming activity. The amino-terminal portion of the Maf protein is rich in acidic residues, and similar acidic regions in other transcriptional factors are often responsible for their transcriptional activator functions (26). Thus, the amino-terminal portion of Maf may also have a transcriptional activator function.

Recently, Swaroop et al. (33) identified a maf-related gene, NRL, which is specifically expressed in human retinal cells. The most striking homology between the v-Maf and NRL proteins resides in the b-Zip domains of these proteins, suggesting that they might recognize common target sequences. Interestingly, the amino-terminal portions of the two proteins also share significant homology. In addition, another maf-related gene, mafB, which we have identified in a CEF cDNA library, also encodes a protein with strong homology with Maf and NRL in the b-Zip domain as well as amino-terminal portion (unpublished data). Thus, the amino-terminal portions of these proteins may play an important role for their biological functions.

The region of v-Maf essential for its basal transforming activity primarily consists of the b-Zip domain. Mutations that alter the b-Zip structure of other b-Zip-type transcriptional regulators are known to alter their DNA-binding activities (9, 17, 21, 27, 28, 35). In the case of Maf, mutations

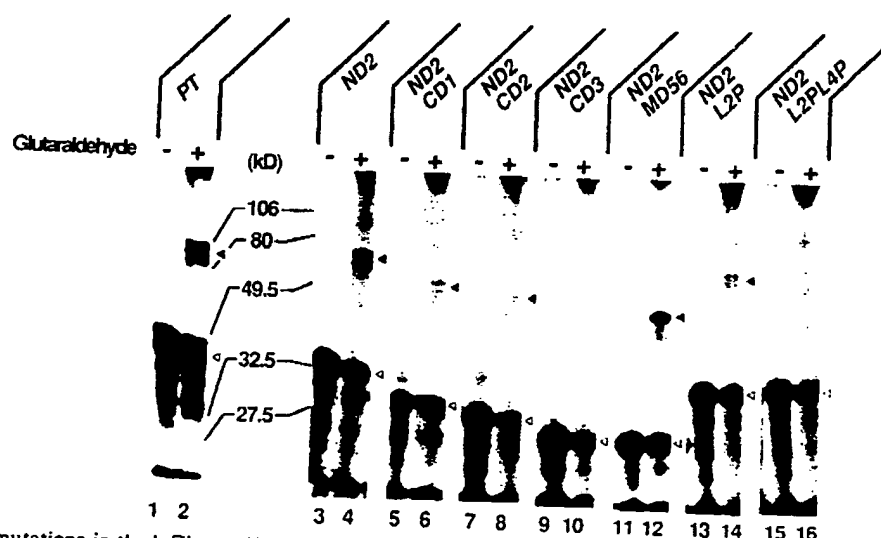


FIG. 8. Effects of mutations in the b-Zip motif on the dimerization of Maf proteins. Positions of the monomer and cross-linked dimer of each mutant protein are indicated by open and closed arrowheads, respectively. Numbers between lanes 2 and 3 indicate molecular sizes.

in the basic, putative DNA binding domain had little effect on its dimer-forming ability, but they completely abolished its transforming activity; this effect was probably due to the lack of DNA-binding activity in these mutant proteins. Mutations in the heptad leucine repeats also affected the dimer-forming ability and transforming activity of the Maf protein. Loss or substitution of one of the leucine residues in the repeat structure was not sufficient to destroy the dimer-forming ability of Maf. This finding is not surprising because Maf contains longer heptad repeats of leucine residues (six repeats) than do most of the other b-Zip proteins (four or five repeats). However, further structural changes, loss of three leucine residues (CD3), or substitutions of two leucine residues with proline residues (L2PL4P) diminished both dimer-forming ability and transforming activity.

We have also shown in this study that overexpression of the *c-maf* proto-oncogene causes cell transformation. In general, viral oncogenes are modified and sometimes truncated versions of their cellular counterparts, and they usually exhibit stronger transforming activities than do their cellular counterparts (2, 22). For instance, even under the control of a strong retroviral promoter, the *c-rel* proto-oncogene exhibits no transforming activity (34). Some of the cellular oncogenes, such as *c-jun*, exhibit some transforming potential when they are overexpressed, but mutations seem to be necessary to convert them to the fully transforming genes (4). The *ski* gene constitutes an interesting exception in that the overexpressed *c-ski* gene is more potent in transforming activity than is its viral counterpart, *v-ski* (6). In the case of *maf*, two structural changes are found between the coding regions of *v-maf* and *c-maf* (unpublished data): (i) a substitution of the methionine residue at position 257 to valine, and (ii) fusion of the viral *gag* sequence to the 5'-terminal end. Neither of these structural changes, however, seems to affect the dimer-forming ability and transforming activity of Maf. Thus, overexpression or escape from a posttranscriptional regulation or both may be important for the transforming potential of the *v-maf* gene. Miller et al. (25) reported that the truncation of 3' noncoding sequences of *c-fos* mRNA, which contains the sequence responsible for rapid mRNA turnover, was important for oncogenic activation. In fact, as does the *c-fos* mRNA, the *c-maf* transcript has relatively long noncoding sequences at both ends (unpublished data).

During the in vitro mutagenesis study, we noted that one point mutation (Q5H) potentiates the transforming activity of v-Maf. At present, we do not know the mechanism of this potentiation, as this mutation has no apparent effects on the dimer-forming ability of v-Maf. On the other hand, we have found in the AS42 virus stock a series of naturally occurring, shorter variants of the *v-maf* oncogene which retain transforming activity comparable to that of the parental *v-maf* gene (12). Lack of accumulation of these various structural alterations in the *v-maf* gene may reflect the recent isolation of the AS42 virus and its cloning prior to multiple passages.

In this study, the v-Maf protein was found to form homodimers in vitro, and no other v-Maf-associated protein could be detected in the lysate of AS42-infected CEF cells by immunoprecipitation with a Maf-specific antiserum, suggesting that the viral Maf protein probably acts as a homodimer. We cannot, however, exclude the possibility that v-Maf forms heterodimers with other leucine zipper proteins. In fact, we have recently found that the product of a *maf*-related gene, *mafB*, forms heterodimers with v-Maf in vitro (unpublished data). The ability of other leucine zipper proteins to form heterodimers with Maf remains to be tested.

The recognition sequence for the DNA binding of Maf also remains to be elucidated. The putative DNA binding domain of Maf shares 20 to 30% similarity with the corresponding region of the Jun and CREB proteins. We could not, however, detect the specific association of Maf protein to TPA- and cAMP-responsive elements, the recognition sequences for Jun or CREB proteins, by gel mobility shift assay (data not shown). This observation suggests that the Jun and Maf proteins transform cells through distinct mechanisms, probably by activating the transcription of different groups of cellular genes. Thus, the search for the DNA binding sequence of the Maf protein is an important effort and is currently under way.

#### ACKNOWLEDGMENTS

We thank Makoto Noda for valuable discussion and critical reading of the manuscript. We are grateful to Shunsuke Ishii and Toshio Maekawa for useful suggestions, Sharad Kumar and Nicholas D. James for careful review of the manuscript, and Tadayuki Takeda for synthesis of oligonucleotides. We also thank Hideko Shinno-Kono and Naomi Nakabayashi for excellent technical assistance.

This work was supported by a research grant from the Princess Takamatsu Cancer Research Fund and by a grant-in-aid for cancer research from the Ministry of Education, Science and Culture of Japan.

#### REFERENCES

1. Benbrook, D. M., and N. C. Jones. 1990. Heterodimer formation between CREB and JUN proteins. *Oncogene* 5:295-302.
2. Bishop, J. M., and H. E. Varmus. 1985. Supplement; function and origins of retroviral transforming genes, p. 249-356. In R. Weiss, N. Teich, H. E. Varmus, and J. Coffin (ed.), *RNA tumor viruses*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. Bohmann, D., T. J. Bos, A. Admon, T. Nishimura, P. K. Vogt, and R. Tjian. 1987. Human proto-oncogene *c-jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* 238:1386-1392.
4. Bos, T. J., F. S. Montecarlo, F. Mitsunobu, A. R. Ball, Jr., C. H. W. Chang, T. Nishimura, and P. K. Vogt. 1990. Efficient transformation of chicken embryo fibroblasts by c-Jun requires structural modification in coding and non-coding sequences. *Genes Dev.* 4:1677-1687.
5. Chiu, R., W. J. Boyle, J. Meek, T. Smeal, T. Hunter, and M. Karin. 1988. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell* 54:541-552.
6. Colmenarejos, C., P. Suttrave, S. H. Hughes, and E. Stavnezer. 1991. Activation of the *c-ski* oncogene by overexpression. *J. Virol.* 65:4929-4935.
7. Curran, T., C. V. Beveren, N. Ling, and I. M. Verma. 1985. Viral and cellular *fos* proteins are complexed with a 39,000-dalton cellular protein. *Mol. Cell. Biol.* 5:167-172.
8. Curran, T., and B. R. Franza, Jr. 1988. Fos and Jun: the AP-1 connection. *Cell* 55:395-397.
9. Gentz, R., F. J. Rauscher, C. Abate, and T. Curran. 1989. Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. *Science* 243:1695-1699.
10. Hai, T., and T. Curran. 1991. Cross-family dimerization of transcriptional factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. USA* 88:3720-3724.
11. Harlow, E., and D. Lane. 1988. *Antibodies: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. Kawai, S., N. Goto, K. Kataoka, T. Saegusa, H. Shinno-Kohno, and M. Nishizawa. 1992. Isolation of the avian transforming retrovirus, AS42, carrying the *v-maf* oncogene and initial characterization of its gene product. *Virology* 188:778-784.
13. Kawai, S., and H. Hanafusa. 1972. Plaque assay for some strains of avian leukosis virus. *Virology* 48:126-135.
14. Kawai, S., and M. Nishizawa. 1984. New procedure for DNA

- transfection with polycation and dimethyl sulfoxide. *Mol. Cell. Biol.* 4:1172-1174.
15. Kawai, S., M. Nishizawa, T. Yamamoto, and K. Toyoshima. 1987. Cell transformation by a virus containing a molecularly constructed *gag-erbB* fused gene. *J. Virol.* 61:1665-1669.
  16. Kawai, S., and T. Yamamoto. 1970. Isolation of different kinds of non-virus producing chick cells transformed by Schmidt-Ruppin strain (subgroup A) of Rous sarcoma virus. *Jpn. J. Exp. Med.* 40:243-256.
  17. Kouzarides, T., and E. Ziff. 1988. The role of the leucine zipper in the fos-jun interaction. *Nature (London)* 346:646-651.
  18. Kozak, M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44:283-292.
  19. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367-382.
  20. Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 240:1759-1764.
  21. Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science* 243:1681-1688.
  22. Lewin, B. 1991. Oncogenic conversion by regulatory changes in transcriptional factors. *Cell* 64:303-312.
  23. Macgregor, P. F., C. Abate, and T. Curran. 1990. Direct cloning of leucine zipper proteins: Jun binds cooperatively to the CRE with CRE-BP1. *Oncogene* 5:451-458.
  24. Marston, F. A. O. 1987. The purification of eukaryotic polypeptides expressed in *Escherichia coli*, p. 59-88. *In* D. M. Glover (ed.), *DNA cloning*, vol. 3. IRL Press, Oxford.
  25. Miller, A. D., T. Curran, and I. M. Verma. 1984. *c-fos* protein can induce cellular transformation: a novel mechanism of activation of a cellular oncogene. *Cell* 36:51-60.
  26. Mitchell, P., and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 289:371-378.
  27. Neuber, M., M. Schuermann, J. B. Hunter, and R. Muller. 1989. Two functionally different regions in Fos are required for the sequence-specific DNA interaction of the Fos/Jun protein complex. *Nature (London)* 338:589-590.
  28. Neuber, M., M. Schuermann, and R. Muller. 1991. Mutagenesis of the DNA contact site in Fos protein: compatibility with the scissors grip model and requirement for transformation. *Oncogene* 6:1325-1333.
  29. Nishizawa, M., K. Kataoka, N. Goto, K. T. Fujiwara, and S. Kawai. 1989. *v-maf*, a viral oncogene that encodes a "leucine zipper" motif. *Proc. Natl. Acad. Sci. USA* 86:7711-7715.
  30. Rauscher, F. J., III, D. R. Cohen, T. Curran, T. J. Bos, P. K. Vogt, D. Bohmann, R. Tjian, and B. R. Franza, Jr. 1988. Fos-associated protein p39 is the product of the *jun* proto-oncogene. *Science* 240:1010-1016.
  31. Rosenberg, A. H., B. N. Lade, D.-S. Chui, S.-W. Lin, J. J. Dunn, and F. W. Studier. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* 56:125-135.
  32. Semba, K., S. Kawai, Y. Matsuzawa, Y. Yamanashi, M. Nishizawa, and K. Toyoshima. 1990. Transformation of chicken embryo fibroblast cells by avian retroviruses containing the human *lyn* gene and its mutated genes. *Mol. Cell. Biol.* 10:3095-3104.
  33. Swaroop, A., J. Xu, H. Pawar, A. Jackson, C. Scolnick, and N. Agarwal. 1992. A conserved retina-specific gene encodes a basic motif/leucine zipper protein. *Proc. Natl. Acad. Sci. USA* 89:266-270.
  34. Sylla, B. S., and H. M. Temin. 1986. Activation of oncogenicity of the *c-rel* proto-oncogene. *Mol. Cell. Biol.* 6:4709-4716.
  35. Turner, R., and R. Tjian. 1989. Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. *Science* 243:1689-1694.
  36. Vinson, C. R., P. B. Sigler, and S. L. McKnight. 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* 246:911-916.
  37. Vogt, P. K. 1964. Fluorescence microscopic observation on the defectiveness of Rous sarcoma virus. *Natl. Cancer Inst. Monogr.* 17:527-541.

# Differential Regulation of IL-12 and IL-10 Gene Expression in Macrophages by the Basic Leucine Zipper Transcription Factor c-Maf Fibrosarcoma<sup>1</sup>

Shanjin Cao,\* Jianguo Liu,\* Marta Chesi,<sup>†</sup> Peter Leif Bergsagel,<sup>†</sup> I-Cheng Ho,<sup>‡</sup> Raymond P. Donnelly,<sup>§</sup> and Xiaojing Ma<sup>2\*</sup>

IL-12 is a principal activator of both innate and adaptive immunity against infectious agents and malignancies. Regulation of proinflammatory IL-12 gene expression in phagocytes by the anti-inflammatory cytokine IL-10 represents a major homeostatic process underlying host-pathogen and host-self interactions. Delineation of the signaling pathway of IL-10 is crucial to the understanding of immunological regulatory networks. In this study, we report that IL-10 and c-musculoaponeurotic fibrosarcoma (Maf) induce their mutual expression in inflammatory macrophages. We demonstrate that c-Maf is one of the physiological mediators of IL-10's immunosuppressive activities. When overexpressed, c-Maf selectively inhibits transcriptional activation of *IL-12 p40* and *p35* genes while potentially activating IL-10 and IL-4 expression, potentially contributing to the development of a state of anti-inflammation and dichotomy of immunologic polarization. c-Maf induces changes in nuclear DNA-binding activities at multiple sites including the ets, GA-12, NF- $\kappa$ B, C/EBP, and AP-1 elements. Nonetheless, the essential c-Maf-responsive element appears to be located elsewhere. Inhibition of *IL-12 p40* gene expression by c-Maf requires the N-terminal transactivation domain, suggesting an indirect mechanism of transcriptional inhibition involving the induction of an unidentified repressor. In c-Maf-deficient murine macrophages, IL-10 production is impaired. However, IL-10-mediated inhibition of IL-12 production remains intact, indicating the existence of alternative mediators in the absence of c-Maf, consistent with the observation that a functional AP-1 is required for this pathway. *The Journal of Immunology*, 2002, 169: 5715–5725.

Inflammatory responses help the immune system respond to infection, while anti-inflammatory responses, arising as a by-product, control excessive damage to the host. Loss of this balance can lead to disproportionate pathology or immunosuppression. Cytokines play major roles in regulating the type, duration, and extent of immune responses in a temporal and spatial manner. IL-10 and IL-12 are two key players in these processes, usually acting in opposition.

IL-12 is a heterodimer produced primarily by macrophages and dendritic cells in both innate and adaptive immune responses and helps induce T cell-dependent and -independent activation of macrophages and NK cells, generation of Th type 1 and cytotoxic T cells, and resistance to intracellular infections (1). IL-12 has powerful antitumor and antimetastatic activities against many murine and human tumors (2). The genes encoding the two heterologous chains of IL-12, *p40*, and *p35* are located on different human chromosomes. The highly coordinated expression of *p40* and *p35* genes

is essential for the initiation of an effective immune response. Infectious agents and tumors evade immune activation by producing immunosuppressive agents such as IL-10, IL-4, TGF- $\beta$ , PGE<sub>2</sub>, glucocorticoids, etc. that inhibit IL-12 production. How these immune suppressants inhibit IL-12 production at the molecular level is not well understood.

We have previously demonstrated that IL-10-mediated inhibition of IL-12 production in human monocytes occurs primarily at the level of transcription of the *p40* and *p35* genes and requires de novo protein synthesis (3). Efforts to delineate the molecular pathway of this inhibition required identification of mediators of IL-10's potent anti-inflammatory and immunosuppressive activities. We used cDNA microarray analysis to search for genes that were induced by IL-10 in LPS-activated human macrophages as candidates to participate in inhibition of IL-12 production. One of the genes we identified in this search was c-musculoaponeurotic fibrosarcoma (Maf).<sup>3</sup>

c-Maf is the cellular counterpart of v-Maf, the transforming gene of the avian retrovirus AS42 for Maf. c-Maf belongs to a growing family of basic leucine zipper (bZIP) transcription factors (4). Along with cyclin D1 and fibroblast growth factor receptor 3, c-Maf was identified as one of three most frequently dysregulated proto-oncogenes by chromosomal translocation to an IgH locus in human multiple myeloma (5, 6). The role of cyclin D1, fibroblast growth factor receptor 3, and c-Maf in the etiology of this malignancy has not been defined. The first direct demonstration of a physiological role of c-Maf was provided by studies in mice genetically rendered deficient in this gene (7–9). Disruption of the

Departments of \*Microbiology and Immunology, and <sup>†</sup>Medicine, Weill Medical College of Cornell University, New York, NY 10021; <sup>‡</sup>Department of Immunology and Infectious Diseases, Harvard School of Public Health, and Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA 02115; and <sup>§</sup>Division of Therapeutic Proteins, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892

Received for publication July 8, 2002. Accepted for publication September 16, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by National Institutes of Health Grants AI-45899 and CA-79772 (to X.M.). S.C. is supported by a Susan G. Komen Breast Cancer Foundation Postdoctoral Fellowship.

<sup>2</sup> Address correspondence and reprint requests to Dr. Xiaojing Ma, Department of Microbiology and Immunology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021. E-mail address: xim2002@med.cornell.edu

<sup>3</sup> Abbreviations used in this paper: Maf, musculoaponeurotic fibrosarcoma; bZIP, basic leucine zipper; RPA, RNase protection assay; RT, reverse transcription; LZ, leucine zipper domain; HO, heme oxygenase; IRES, internal ribosomal entry site; EGFP, enhanced green fluorescent protein.

c-Maf gene affected both intrauterine and postnatal survival (7). Subsequently, it was shown that deficiency in c-Maf resulted in a specific defect of IL-4 production by CD4<sup>+</sup> T lymphocytes and a lack of Th2 differentiation (10). Thus, c-Maf is both a developmentally and immunologically important gene.

In this study, we demonstrate that c-Maf is a potent activator of IL-10 gene expression in monocytes/macrophages. When overexpressed, it could also suppress IL-12 p40 and p35 gene transcription. We explore the underlying molecular mechanisms.

## Materials and Methods

### Cells and reagents

Human monocytes were obtained by leukopheresis and the purity of the preparations was routinely >95%. The murine monocyte-like cell line RAW264.7 was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, and penicillin/streptomycin. Anti-IL-10 and isotype control Abs were purchased from R&D Systems (Santa Cruz, CA). Recombinant human and murine IFN- $\gamma$  were purchased from Genzyme (Boston, MA). Recombinant human M-CSF was purchased from PeproTech (Rocky Hill, NJ). LPS from *Escherichia coli* 0127:B8 was purchased from Sigma-Aldrich (St. Louis, MO). All Abs used in EMSA analysis were from Santa Cruz Biotechnologies (Santa Cruz, CA).

### Microarray analysis

The Atlas Human 1.2 Array (catalog no. 7850-1; Clontech Laboratories, Palo Alto, CA) contains 1176 human genes, nine housekeeping control cDNAs, and negative controls immobilized on a nylon membrane. The manufacturer's instructions were followed for probe synthesis, hybridization, washing, signal scanning (in PhosphorImager Storm 860; Molecular Dynamics, Sunnyvale, CA), and data analysis. Quantitative data analysis was performed using the PhosphorImage software ImageQuant 5.0. RAW data were normalized between membrane pairs by global means.

The Affymetrix (Santa Clara, CA) oligonucleotide array HG-U95A contains 12,600 sequences (each represented by 16 pairs of 25-mer oligonucleotides). Manufacturer's instructions were followed in the use of these arrays. Data analysis of the array data was performed using Microarray Suite (Affymetrix) and GeneSpring (Silicon Genetics, Redwood City, CA).

### Plasmids

All human IL-12 p40 promoter-luciferase constructs have been described previously (11, 12). The human IL-12 p35 promoter was as described (13). The murine IL-4 promoter-luciferase construct was obtained from Dr. R. Flavell of Yale University (New Haven, CT; Ref. 14). The human IL-10 promoter-luciferase construct was obtained from Dr. L. Ziegler-Heitbrock of University of Leicester (Leicester, U.K.). It contains a piece of the human IL-10 promoter region up to -1044. The NF- $\kappa$ B luciferase plasmid was purchased from Stratagene (La Jolla, CA; catalog no. 219078). The human c-Maf cDNA (both long and short isoforms) was tagged with hemagglutinin and cloned into the mammalian expression vector pCEFL, under the EF-1 $\alpha$  promoter (15). The mutant c-Maf construct bZIP domain (LZ) was generated by PCR (sense primer: GGGGAATTCCTGCATTCGAC GACCGCTTC; antisense primer: CCTCTAGATCACATGAAAGCT CGGGAGAGGA). It contains the basic DNA-binding domain and the LZ, lacking the transactivation domain completely. Likewise, the LZ mutant contains only the LZ generated with the sense primer (ACGAATCCA CGTCCTGGAGTCGGAG) and antisense primer (CGTCTAGATCATT TTGTGAACACACTGGT). The C/EBP and AP-1 mutants of the human IL-12 p40 promoter were created by overlapping PCR in the context of the -292/+108 construct. The wild-type sequence in this region is GTTTT CAATGTTGCAACAAGTCAGTT, the C/EBP mutant sequence is GTT TCAATGGACGTCGAAGTCAGTT, with the underlined sequence being the target site different between the two. The AP-1 mutant construct has the sequence of CAACAAGTTGGTTTCTAG vs the wild-type CAAC AAGTCAGTTTCTAG. All mutant constructs generated by PCR were completed sequence verified. The dominant negative mutant of AP-1 (A-Fos) was generously provided by Dr. C. Vinson (National Cancer Institute, National Institutes of Health, Bethesda, MD). All plasmids were purified using the Qiagen Endotoxin free kit (Qiagen, Valencia, CA).

### Adenoviral vectors and their propagation

We integrated p-internal ribosomal entry site 2 (pIRES2)-enhanced green fluorescent protein (EGFP) (Clontech Laboratories) into pAdeno-X express

cDNA (hc-Maf) from its original vector into the EcoRI and SmaI sites in pIRES-EGFP, then transferred the hc-Maf-IRES-EGFP fragment (SacI blunt and XbaI) into pShuttle vector (NheI blunt and XbaI). Finally, the human c-Maf and EGFP coexpression cassette (hc-Maf-IRES-EGFP) was ligated with the pAdeno-X backbone by I-CeuI and PstI-SacI digestion. At the same time, we constructed an EGFP alone expression vector as a control by subcloning the IRES-EGFP sequence into pShuttle vector by NheI and NotI digestion, then transferred the EGFP alone expression cassette into pAdeno-X using the same construction strategy as described for the hc-Maf-IRES-EGFP vector. Viruses were propagated in the human embryonic kidney 293 cell line and purified by ultracentrifugation through two cesium chloride gradients. Titers of viral stocks were determined by plaque assay in human embryonic kidney 293 cells after exposure to virus for 1 h in serum-free DMEM. Freshly isolated human monocytes were cultured in human M-CSF for 2 days. The resulting macrophages were exposed to recombinant virus (200 PFU/cell) for 4–8 h in serum-free RPMI 1640 medium followed by equal volume of RPMI 1640 supplemented with 10% FCS for an additional 24 h. The transfection medium was replaced with fresh RPMI 1640 medium with 10% FCS. Twenty-four hours later, the transfection efficiency was monitored by  $\beta$ -galactosidase staining (16) or EGFP expression under reverse-phase fluorescence microscope.

To construct the lacZ-based adenovirus vectors, Clontech Adeno-X Expression system (Clontech Laboratories) was used. We subcloned human c-Maf cDNA into the pShuttle vector, then subcloned the human c-Maf expression cassette into Adeno-X Viral DNA (Clontech Laboratories). Ad/lacZ was constructed by subcloning lacZ expression cassette from pShuttle/lacZ (supplied in the Clontech Adeno-X Expression system as a positive control) into Adeno-X Viral DNA.

### Transfections

Transient transfections were performed by electroporation as previously described (11). Transfection efficiency was routinely monitored by  $\beta$ -galactosidase assay by cotransfection with 3  $\mu$ g of pCMV- $\beta$ -galactosidase plasmid. Variability between samples was typically <10%. Lysates were used for both luciferase and  $\beta$ -galactosidase assays.

### Cytokine assays

Cytokine secretion was measured by ELISA, using appropriately diluted culture supernatants. Human IL-12 p40 and p70, mouse IL-12 p40, p70, and IL-10 were measured by the respective ELISA kits from BD Pharmingen (San Diego, CA).

### RNase protection assay (RPA)

RPAs were performed using the human CK2b RiboQuant Multiprobe RPA system from BD Pharmingen according to the manufacturer's instructions. A total of 10  $\mu$ g of RNA was used for each determination. The riboprobe for c-Maf was generated by transferring c-Maf cDNA from pCEFL into PCR2.1 (Invitrogen, Carlsbad, CA). For in vitro transcription using T7 RNA polymerase, the plasmid was linearized first with BglII. The resulting probe was 215 bases long, and the protected probe was 125 bases.

### RT-PCR

Reverse transcription (RT) reactions were conducted as follows: 0.4  $\mu$ g total RNA was mixed with 2  $\mu$ l oligo(dT) primers (16 mer, 0.5 mg/ml) and ddH<sub>2</sub>O to equalize volumes of all samples at 8.5  $\mu$ l. The mix was boiled for 5 min, quenched on ice, spun down briefly, and 11.5  $\mu$ l of a Master Mix was added. The RT Master Mix consisted of 4  $\mu$ l 5 $\times$  first strand buffer (Life Technologies, Grand Island, NY), 4  $\mu$ l 2.5 mM dNTPs, 2  $\mu$ l 0.1 M DTT, 0.5  $\mu$ l RNase inhibitor (40 U/ $\mu$ l; Boehringer-Mannheim, Indianapolis, IN), and 1  $\mu$ l Superscript II RT (200 U/ $\mu$ l; Life Technologies). The reaction was incubated at 37°C for 90 min, then 95°C for 10 min, followed by a 4°C soak. To each sample (in 20  $\mu$ l total volume) 80  $\mu$ l ddH<sub>2</sub>O were added. A total of 2.5  $\mu$ l were used for each PCR of 25  $\mu$ l.

The following primers were used for PCR amplification: 1) CCR2 (U03905), upper: CACAGGGCTGTATCACATCG, lower: CCAGTTGA CTGGTGCTTTCA; 2) glutaredoxin (X76648), upper: GCCCAAGAG ATCCTCAGTCA, lower: CAATTGGGTCTGTGACCTT; 3) heme oxygenase (HO)-1 (X06985), upper: ATGACACCAAGGACGAGC, lower: AGACAGCTGCCACATTAGGG; 4) IL-1R type 2 (X59770), upper: TGGGTCTCAGTCTCCACTT, lower: TACCCAGAGGTTGAC AAGG; 5) c-Jun N-terminal kinase 2 (L31951), upper: CCGTCTTTT CAGAACCAAA, lower: CAACCTTTCCAGGCTCTCC; 6) MAPK p38 (L35253), upper: GACACAAAACGGGGTTACG, lower: TGCATCC CACTGACCAATA; 7) hypoxanthine phosphoribosyltransferase (M26434.1), upper: CCTGCTGGATTACATCAAGCACTG, lower: TC

### Nuclear extraction

**EMSA**

#### Generation of macrophages from the fetal liver of c-Maf knockout mice and genotyping

Determination of the genotype of each embryo was performed by PCR using genomic DNA derived from a hind leg and primers that were able to differentiate the wild-type c-Maf gene from the disrupted copy (7).

### Western blotting

Western blot was performed as previously described (18).

### Statistical analysis

Student's *t* test was used for data analysis where appropriate. Data are expressed as mean  $\pm$  SD unless otherwise indicated.

## Results

### *c-Maf* gene expression is induced by IL-10 and IL-4 in activated human peripheral blood monocytes

**a**

**b**

**c**

media	LPS	IFN $\gamma$ /LPS	Cell treatment
IL-10	IL-6	IL-10 IL-6	c-Maf
			IL-12p35
			IL-12p40
			IL-10
			IL-1 $\alpha$
			IL-18
			IL-1Ra
			IL-6
			L32
			GAPDH

**d**

IFN $\gamma$	-	-	-	+	+
LPS	-	+	+	+	+
IL-10	-	-	+	-	+

**e**

IFN $\gamma$ /LPS	-	+	+	+
IL-10	-	-	1h	2h

**FIGURE 1.** Differential gene expression in activated human monocytes treated with IL-10. Elutriated human monocytes were stimulated with LPS (1  $\mu\text{g/ml}$ ) for 4 h or pretreated with recombinant human IL-10 (20 ng/ml) for 2 h followed by LPS stimulation. Some monocytes were pretreated with IFN- $\gamma$  (10 ng/ml) for 16 h followed by IL-10 and LPS treatment. *a* and *b*, cDNA microarray analysis. Total RNA were isolated and 10  $\mu\text{g}$  were labeled by RT in the presence of [ $\alpha$ - $^{32}\text{P}$ ]dATP. An equal number of cpm's of the two probes were applied to hybridization with the Atlas arrays following the manufacturer's instructions. *a*, LPS- vs IL-10 + LPS-treated cells. *b*, IFN- $\gamma$ /LPS- vs IL-10 + IFN- $\gamma$ /LPS-treated cells. Arrows indicate some of the well-known inflammatory cytokines as well as c-Maf. *c*, Correlation of mRNA expression between IL-10-induced c-Maf and IL-12 p40/p35 in human monocytes. Total RNA were subjected to RPA using the BD PharMingen's Multiprobe set hCK2b. The same RNA samples were also analyzed by RPA using a riboprobe for human c-Maf (see *Materials and Methods* for details). *d*, c-Maf protein expression in the nucleus. Human monocytes were stimulated as indicated with LPS or IFN- $\gamma$  plus LPS in the presence or absence of IL-10 (20 ng/ml). Nuclear extracts were isolated and 13  $\mu\text{g}$  of proteins analyzed by denaturing Western blot with a polyclonal anti-c-Maf Ab. The blot was subsequently stripped and reprobed with an anti-PU.1 Ab to assess protein loading. *e*, c-Maf protein expression in the nucleus. Mouse peritoneal macrophages were elicited by thioglycolate injection. Cells were stimulated or not with IFN- $\gamma$  plus LPS in the presence or absence of IL-10 (20 ng/ml) for 1 or 2 h. Nuclear extracts were isolated and 34  $\mu\text{g}$  of proteins analyzed by denaturing Western blot with the same polyclonal anti-c-Maf Ab used above, which reacts with both human and murine c-Maf.

group of genes that were induced >4-fold in monocytes from five of five donors treated with LPS or IFN- $\gamma$  plus LPS and IL-10 are listed in Table I. Several of these genes have been implicated in anti-inflammatory or Th2 responses (20–26). Among these, c-Maf caught our attention because of its role in promoting IL-4 gene expression and Th2 development, a process that functionally opposes IL-12-induced Th1 differentiation (10).

We verified by RT-PCR the status of differential expression of some of the genes identified in this search using RNA isolated from monocytes of a separate donor with the same stimulations. All genes appeared to be constitutively expressed, and the constitutive expression was inhibited in monocytes activated by LPS or IFN- $\gamma$  plus LPS. Treatment of activated cells with IL-10 significantly up-regulated the mRNA expression of these genes (data not shown). The differential expression of c-Maf mRNA was confirmed by RPA, which revealed an inverse relationship with IL-12 p35 and p40 mRNA expression (Fig. 1c). Notably, c-Maf mRNA expression in monocytes was also constitutive (lane 1). LPS or IFN- $\gamma$  plus LPS challenge of monocytes resulted in its suppressed expression (lanes 2 and 5). Both IL-10 and IL-4 treatment of LPS-stimulated monocytes caused an induction of c-Maf mRNA (lanes 3 and 4), but IL-4 failed to induce c-Maf in IFN- $\gamma$ /LPS-treated cells, and to inhibit IL-12 p35 and p40 expression (lane 7), suggesting that the mechanisms of induction of c-Maf expression by IL-10 and IL-4 are likely different.

Western blot analyses were also performed to evaluate the regulation of nuclear c-Maf protein production by IL-10 in human monocytes and mouse peritoneal macrophages. In human monocytes, c-Maf was constitutively present in the nucleus (Fig. 1d, lane 1). Upon LPS or IFN- $\gamma$  plus LPS stimulation, c-Maf level was strongly reduced (lanes 2 and 4), whereas IL-10 treatment reversed this inhibition (lanes 3 and 5). This result is consistent with the mRNA data presented in Fig. 1d. In thioglycolate-elicited mouse peritoneal macrophages, c-Maf protein expression was also constitutive (Fig. 1e, lane 1). However, cellular activation by IFN- $\gamma$  and LPS treatment did not result in a complete down-regulation of

c-Maf (lane 2). IL-10 treatment for 1 or 2 h led to a strong up-regulation of c-Maf in these cells (lanes 3 and 4).

#### c-Maf expression in primary macrophages selectively inhibits IL-12 gene expression and induces IL-10 expression

To determine whether c-Maf expression in PBMC-derived human macrophages was causative for suppressed IL-12 production, we transduced human monocyte-derived macrophages obtained by culturing in M-CSF with an adenovirus carrying either a cDNA coding for human c-Maf or for the lacZ gene. As shown in Fig. 2a, macrophages transduced with a lacZ-expressing virus produced IL-12 p40 (upper panel) and p70 (middle panel) when stimulated with LPS alone (p40 only) or IFN- $\gamma$  plus LPS. Culturing monocytes in M-CSF resulted in a "priming" effect for expression of IL-12 p40 but not p70, i.e., p40 production no longer depended on IFN- $\gamma$ . Transduction of macrophages with the c-Maf-expressing virus caused a strong inhibition of IL-12 p40 and p70 secretion. However, IL-10 production was induced by c-Maf in resting macrophages, and markedly enhanced in LPS or IFN- $\gamma$  plus LPS-stimulated cells (lower panel).

We next addressed the question of whether the inhibitory effects of c-Maf were restricted to IL-12. We transduced human macrophages with Ad/EGFP or Ad/c-Maf, and analyzed cytokine mRNA expression by RPA in these cells following appropriate stimulation

Table I. Genes that were significantly induced in LPS-activated human monocytes by IL-10<sup>a</sup>

Gene	Accession No.	Function or Relevance
ADP-ribosylation factor 1	M36340	Hydrolize GTP to GDP in vesicular transport
CCR2	U03905	Chemokine receptor induced by IL10 in LPS macrophage
c-Maf	AF055377	Th2 transcription factor, protooncogene
Glutaredoxin	X76648	Carry electrons from NADPH to ribonucleotide reductase; Important for protection against oxidative stress
HO-1	X06985	First step in degradation of heme to bilirubin and CO (anti-inflammatory)
IL-1R type II	X59770	Mediate Th2 proliferation and cytokine production
JNK2 <sup>b</sup>	L31951	Involved in NF-AT-dependent transcription, papilloma
MAPK p38	L35253	Mediate IL10-dependent Th2 suppression in sepsis

<sup>a</sup> The genes identified in this search are selected that satisfy all the following four criteria: 1) signal levels are >1000 in the IL-10-treated samples; 2) fold of change is >4 in IL-10-treated cells; 3) consistently induced in all five donors tested; 4) IL-12 production was inhibited in all IL-10-treated samples.

<sup>b</sup> JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

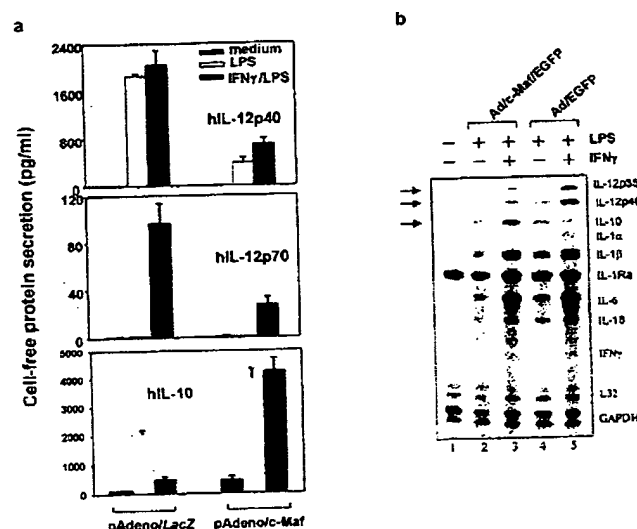


FIGURE 2. c-Maf is an inhibitor of IL-12 protein secretion and mRNA expression by macrophages. *a*, Adenovirus/c-Maf-mediated inhibition of IL-12 production in human macrophages. Human monocyte-derived macrophages were transduced with adenovirus/c-Maf or Ad/lacZ at 200 PFU/cell for 8 h. Cells were subsequently washed and stimulated with LPS alone (24 h) or IFN- $\gamma$  (16 h) followed by LPS (24 h). Cell-free supernatant for assayed by ELISA for the production of human IL-12 p40 (upper panel), p70 (middle panel), and IL-10 (lower panel). The data shown in this figure are derived from one of three independent experiments with very similar results. The rate of adenovirus transduction in this experiment was between 20 and 30%. To normalize the data from the variously transduced cells, total cellular protein contents were measured and they showed little variation among samples. *b*, Differential effects of c-Maf on cytokine mRNA expression in human macrophages. Human peripheral blood-derived macrophages were transduced with Ad/GFP or Ad/c-Maf/GFP for 24 h followed by stimulation with IFN- $\gamma$  for 16 h followed by LPS stimulation for 4 h. Total RNA was isolated and multiple cytokine RPA performed using the hCK2b probe set. The three arrows highlight the three inversely affected genes by c-Maf expression: IL-12 p35, p40, and IL-10, respectively. One representative RPA of three is shown.



with IFN- $\gamma$  and LPS (Fig. 2b). c-Maf expression in these macrophages resulted in a strong inhibition of mRNA expression of IL-12 p40 and p35 with minimal effect on other cytokines and a strong induction of IL-10 mRNA synthesis, confirming the protein profiles of these cytokines (Fig. 2a). This indicates that c-Maf inhibits IL-12 gene expression selectively, and c-Maf and IL-10 could induce each other's expression.

The observation of the ability of c-Maf to up-regulate IL-10 expression in inflammatory macrophages prompted the question of whether c-Maf-mediated inhibition of IL-12 production was dependent on IL-10. To address this possibility, we applied neutralizing IL-10 Ab to the cultures of c-Maf-transduced human macrophages. In two of three donors, the inhibition of IL-12 p40 or p70 production by the transduced c-Maf expression was not reversed at all by the presence of the Ab while there was a partial reversal in the third donor (data not shown). Nonetheless, in all three donors c-Maf transduction resulted in strong inhibition of IL-12 production. The lack of a consistent correlation between blocking of IL-10 in c-Maf-transduced macrophages and a reversal of c-Maf-mediated inhibition of IL-12 production led us to conclude that c-Maf does not inhibit IL-12 production simply by stimulating IL-10 production.

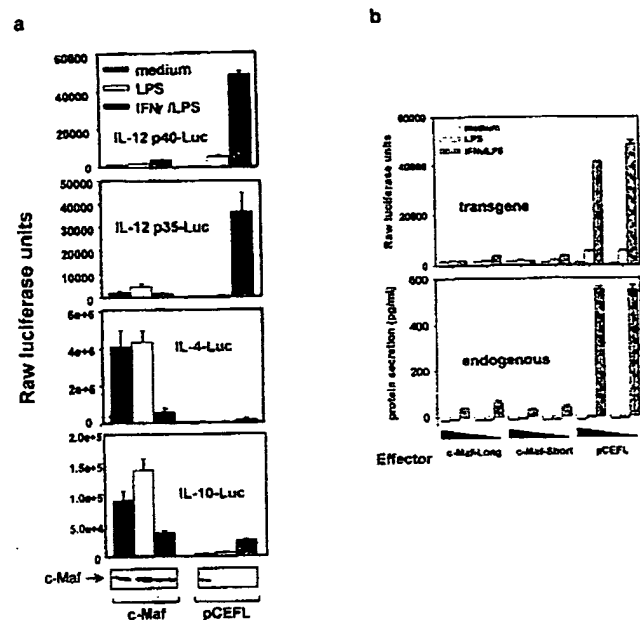
#### c-Maf differentially regulates IL-12 p35, IL-12 p40, and IL-4 and IL-10 gene transcription

To further delineate the molecular mechanism by which c-Maf exerts its inhibitory effects on IL-12 p40 and p35 transcription, we used a well-established transient transfection system in the murine monocytic cell line RAW264.7 (11). When the human IL-12 p40, p35, or IL-4 and IL-10 promoter-luciferase reporter constructs were cotransfected into RAW cells, IL-12 p40 and p35 promoter-driven luciferase activity in IFN- $\gamma$ /LPS-stimulated cells was strongly inhibited by c-Maf expression (Fig. 3a). In contrast, IL-4 and IL-10 promoter activities were greatly enhanced, consistent with the reported selective role of c-Maf in IL-4 gene transcription (14). Notably, IFN- $\gamma$  strongly suppressed c-Maf-induced IL-4 and IL-10 transcription.

It is generally believed that in transient transfections, the reporter gene (herein termed "transgene") is not associated with a chromatin structure as is the endogenous gene. To ascertain if the observed effects of c-Maf on the IL-12 p40 reporter gene in RAW cells could also pertain to the endogenous (chromosomal) IL-12 p40 gene, we cotransfected the human IL-12 p40 promoter-reporter construct with the control vector pCEFL or the two isoforms of c-Maf (long and short; Ref. 5) in two amounts (reporter to effector molar ratio of 3:1 and 9:1, respectively). Both IL-12 p40 promoter-driven luciferase activity and the endogenous IL-12 p40 protein secretion in RAW cells stimulated by IFN- $\gamma$  and LPS were strongly inhibited as a result of c-Maf expression (Fig. 3b), indicating that the transcription of IL-12 p40 is suppressed by c-Maf acting on the "episomal" and chromosomal genes alike.

#### Localization of the c-Maf-responsive element within the IL-12 p40 promoter

The human IL-12 p40 promoter contains three critical cis-elements involved in the regulation of its transcription by LPS and IFN- $\gamma$ : an ets site at -211/-206 (TTTCCT), an "NF- $\kappa$ B half site" at -117/-107 (TGAAATCCCC), and a C/EBP site at -72/-80 (ATGTTGCAA). The ets site and its surrounding sequences interact with a large complex named F1, which is induced by either LPS or IFN- $\gamma$ , and is composed of ets-2, PU.1, IFN regulatory factor-1, IFN consensus binding protein, NF- $\kappa$ B c-Rel, and a novel ets-2-related protein (11, 12, 27). The NF- $\kappa$ B half site binds p50/p65 and p50/c-Rel heterodimers induced by LPS (12, 28-30). The



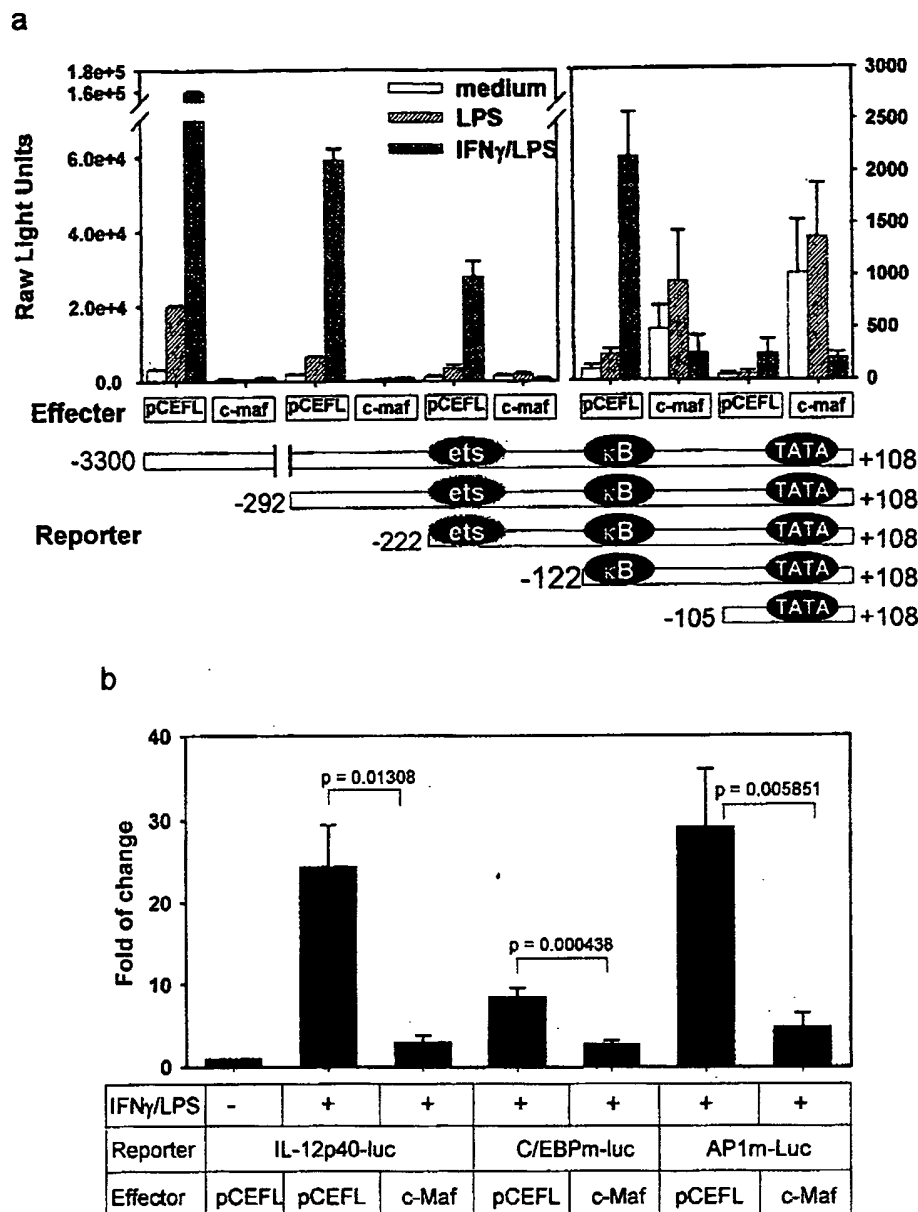
**FIGURE 3.** c-Maf differentially regulates IL-12 p35, p40, IL-4, and IL-10 transcription. *a*, c-Maf-mediated regulation of IL-12 p40, p35, IL-4, and IL-10 reporter gene transcription in RAW264.7 cells. Human IL-12 p40 (3.3 kb), p35 (1.14 kb), mouse IL-4, or human IL-10 promoter-luciferase constructs were cotransfected transiently with a human c-Maf expression vector or its empty parental vector pCEFL into RAW264.7 cells at a molar ratio of 3:1 (reporter:effector). Cells were then stimulated with LPS alone or IFN- $\gamma$  (16 h) followed by LPS (7 h). Cell lysates were prepared and assayed for luciferase activity using a luminometer. The data are summaries of three independent experiments. The protein expression of transfected c-Maf (human) in this transient transfection was analyzed by Western blot using a polyclonal anti-c-Maf Ab that cross-reacts with both human and mouse c-Maf. Note the constitutive c-Maf expression in c-Maf-transfected cells, and an absence of such expression in control vector-transfected cells stimulated by LPS or IFN- $\gamma$  plus LPS. The c-Maf expression in unstimulated cells was derived from the endogenous gene (mouse). *b*, c-Maf-mediated inhibition of the endogenous (chromosomal) IL-12 p40 gene expression in RAW264.7 cells. The 3.3-kb human IL-12 p40 promoter-luciferase construct was cotransfected transiently with a c-Maf expression vector (carrying the cDNA for the long or short isoform of c-Maf) or the control vector pCEFL into RAW264.7 cells at a molar ratio of 3:1 or 9:1 (reporter:effector). The luciferase activity derived from this IL-12 p40 promoter is defined as that of the transgene. Cells were then stimulated with LPS alone or IFN- $\gamma$  (16 h) followed by LPS (7 h). Cell lysates were prepared and assayed for luciferase activity using a luminometer. Cell-free supernatants were assayed by ELISA to measure the endogenous mIL-12 p40 secretion, i.e., that derived from the chromosomal gene. The results are summaries of three independent experiments.

C/EBP site interacts with members of the C/EBP family, particularly C/EBP $\beta$  (31). Another motif recently described as a negative element in the IL-12 p40 promoter is the GA-12 site (GATA) located at -157/-160. The binding activity of the GA-12 binding protein GAP-12 was increased by treatment with two inhibitors of IL-12 expression, IL-4 and PGE2 in human CD14<sup>+</sup> monocytes. Moreover, IL-4-mediated repression of IL-12 p40 promoter activity was critically dependent on an intact GA-12 sequence (32).

To identify the promoter element(s) through which c-Maf mediates its inhibitory effects on IL-12 p40 transcription, we used various deletion mutants of the p40 promoter-luciferase constructs, and cotransfected them with the c-Maf expression vector or the control vector into RAW264.7 cells. As shown in Fig. 4a, deletion of the 3300-bp p40 promoter to -222 reduced the overall promoter activities under the three experimental conditions but did not



**FIGURE 4.** Localization of the c-Maf-responsive element in the human *IL-12 p40* promoter. **a**, A series of 5' deletion mutants of the full-length human *IL-12 p40* promoter-luciferase construct, which spans 3.3 kb upstream and 108 bp downstream of the transcription initiation site, were cotransfected transiently with a c-Maf expression vector or the control vector pCEFL into RAW264.7 cells at a molar ratio of 3:1 (reporter:effector). Cells were then stimulated with LPS alone or IFN- $\gamma$  (16 h) followed by LPS (7 h). Cell lysates were assayed for luciferase activity. The data are summaries of four separate experiments. **b**, Effect of mutations at the C/EBP and AP-1 sites. Mutant promoter luciferase constructs of *IL-12 p40* promoter in the context of -292/+108 were cotransfected with c-Maf at a molar ratio of (1:1). Luciferase activity was measured from cell lysates following stimulation of RAW264.7 cells with IFN- $\gamma$  and LPS, and normalized to the medium condition of the wild-type *IL-12 p40* construct, which was taken as 1. Data represent mean plus SD of three independent experiments with duplicate measurements each.



affect the c-Maf-mediated inhibition of the IFN- $\gamma$ /LPS-induced transcription. Further deletion of the promoter to -122, which eliminated the *ets* (11) and GA-12 (32) sites but retained the NF- $\kappa$ B site, drastically reduced the overall promoter activity (note the different scales of luciferase activity used), as reported previously (11), but did not block the response to c-Maf inhibition of IFN- $\gamma$ /LPS-induced reporter activity. Removal of the NF- $\kappa$ B site by deleting a further 17 bp down to -105 resulted in almost complete loss of the inducibility of the promoter by IFN- $\gamma$  and LPS, making it difficult to assess the ability of c-Maf to suppress its activity. Thus, the putative c-Maf-responsive element is either overlapping with the NF- $\kappa$ B site or located further downstream. Of note, the -122 and -105 constructs, as well as a construct that contains only the TATA box (data not shown), consistently responded positively to c-Maf expression in unstimulated or LPS-stimulated cells. This reinforces the notion that c-Maf may also act as a transcriptional activator, depending on the promoter context.

Because of the noted role of the C/EBP (31) and AP-1 (33) sites in the regulation of the mouse *IL-12 p40* promoter, we sought to

hibition of the human *IL-12 p40* transcriptional induction. Base substitutions were introduced into these two sites separately by site-directed mutagenesis in the context of the -292/+108 *IL-12 p40*-luc construct. Cotransfection of these constructs with c-Maf was performed (Fig. 4b). Mutation of the C/EBP site resulted in a substantial reduction of the human *IL-12 p40* promoter activity induced by IFN- $\gamma$  and LPS, confirming the previously reported finding (31). However, c-Maf expression still caused a significant inhibition of the mutant promoter activity. In contrast, the AP-1 mutant did not affect the *IL-12 p40* transcription (see Discussion for an explanation), nor did it affect c-Maf's ability to suppress the induced *p40* promoter activity. Taken together, we conclude that c-Maf's inhibitory effects on *IL-12 p40* promoter activation are not likely mediated through these two sites.

#### Role of the NF- $\kappa$ B site in c-Maf-mediated inhibition of *IL-12 p40* transcription

Next, we focused more closely on the NF- $\kappa$ B site. We cotransfected the -222 wild-type construct with the c-Maf expression

construct in which base substitutions were introduced into the NF- $\kappa$ B site (12). This mutant construct, compared with the -105 construct, exhibits a dramatically reduced ( $\sim 10$ -fold lower) but still measurable promoter activity induced by IFN- $\gamma$  and LPS (12). Fig. 5a shows that while the wild-type -222 and its NF- $\kappa$ B mutant constructs had rather different transcriptional potentials induced by IFN- $\gamma$  and LPS, c-Maf expression in RAW cells nevertheless strongly inhibited both constructs' activities. This indicated that an intact NF- $\kappa$ B response element is not required for c-Maf to exert its suppression on the *p40* promoter. This interpretation is further supported by the observation that the transcriptional activity of an NF- $\kappa$ B-driven luciferase construct was only partially inhibited by c-Maf expression in RAW cells either unstimulated or stimulated with IFN- $\gamma$  and LPS, and not inhibited at all in LPS-stimulated cells. In the same experiments, the full-length *IL-12 p40* promoter activity was totally ablated under all three conditions (Fig. 5b). These results imply that the c-Maf-response element may be located downstream of the NF- $\kappa$ B site.

#### Forced c-Maf expression induces changes in multiple nuclear DNA-protein complexes

We rationalized that if the transcriptional inhibition of *IL-12 p40* mediated by c-Maf should be manifested in the DNA binding activities, it would induce on the *p40* promoter either directly (involving c-Maf binding to the *p40* promoter) or indirectly (without c-Maf binding to *p40* promoter). We performed EMSAs to examine physical DNA-protein interactions following forced c-Maf expression at the ets, GA-12, NF- $\kappa$ B, C/EBP, and AP-1 sites that have been shown to be involved in the regulation of *IL-12 p40* transcription in several systems (11, 28, 31–33). Fig. 6 shows that forced c-Maf expression in RAW264.7 cells by transfection did induce several changes in these binding activities. Most notably, c-Maf blocked the PU.1<sup>+</sup> complex (no. 1; see also supershift in Fig. 6b) identified as a target in Fc $\gamma$ R-mediated inhibition of *IL-12 p40* transcription (13). The difference is that in the Fc $\gamma$ R-induced

change, both PU.1<sup>+</sup> (no. 1) and PU.1 (no. 2) were affected, whereas in c-Maf-mediated alterations, only PU.1<sup>+</sup> complex is abrogated. Another site at which significant changes were seen following c-Maf expression is the NF- $\kappa$ B element. c-Maf expression induced, not inhibited, stronger binding at this site. Supershift analysis indicated that c-Maf-enhanced NF- $\kappa$ B complex was qualitatively similar to that induced in the absence of forced c-Maf expression, and consisted of p50, p65, and c-Rel (Fig. 6b). Binding to the GA-12 element was constitutive in unactivated cells and was reduced following IFN- $\gamma$  and LPS stimulation, consistent with a negative role this site plays in IL-4-mediated inhibition of *IL-12 p40* transcription (31). However, c-Maf expression induced a reduction in this constitutive binding, thus diminishing the difference between IFN- $\gamma$ /LPS-stimulated cells expressing or not expressing c-Maf. Binding activities at the C/EBP/AP-1 site were generally increased by c-Maf.

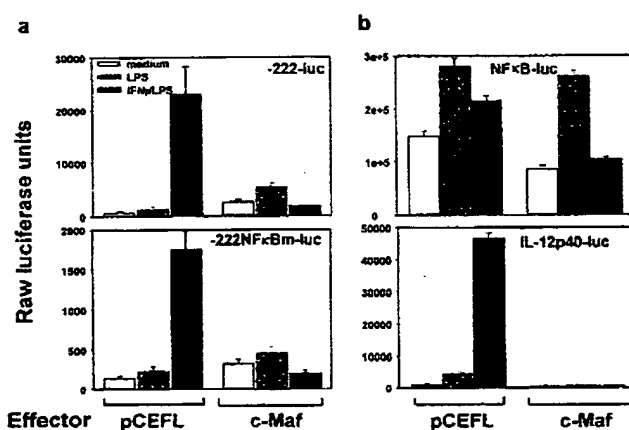
Taken together with the transfection data, these results suggest that c-Maf expression induces changes in multiple nuclear binding activities, that in part explain why in some *p40* deletion constructs, the constitutive promoter activity was enhanced by c-Maf. Although these changes do not seem essential because in their absence, c-Maf is still able to suppress the *p40* promoter stimulated by IFN- $\gamma$  and LPS.

#### Inhibition of *IL-12 p40* transcription and activation of *IL-10* transcription by c-Maf requires its N-terminal transactivation domain

A functional c-Maf consists of an N-terminal transactivation domain, a central, basic DNA-binding domain, and a C-terminal leucine zipper dimerization domain. To determine the requirement of these domains in the inhibition of *IL-12 p40* transcription, we made two constructs of c-Maf with sequential deletions from the N terminus such that it contained no transactivation domain, but retained the DNA-binding and LZs (basic-LZ), or one that contained the LZ only. The ability of these deletion constructs of c-Maf to suppress *IL-12 p40* transcription and to activate the *IL-10* promoter was tested by transient transfection assay in RAW264.7 cells. As shown in Fig. 7a, neither mutant construct was able to inhibit *IL-12 p40* transcription stimulated by IFN and LPS or activate *IL-10* transcription induced by LPS to the degree attained by the full-length construct despite more or less equivalent expression levels of their respective proteins in the nucleus (Fig. 7b), suggesting that the N-terminal transactivation domain is required for c-Maf to play its negative and positive role for *IL-12 p40* and *IL-10* transcription, respectively.

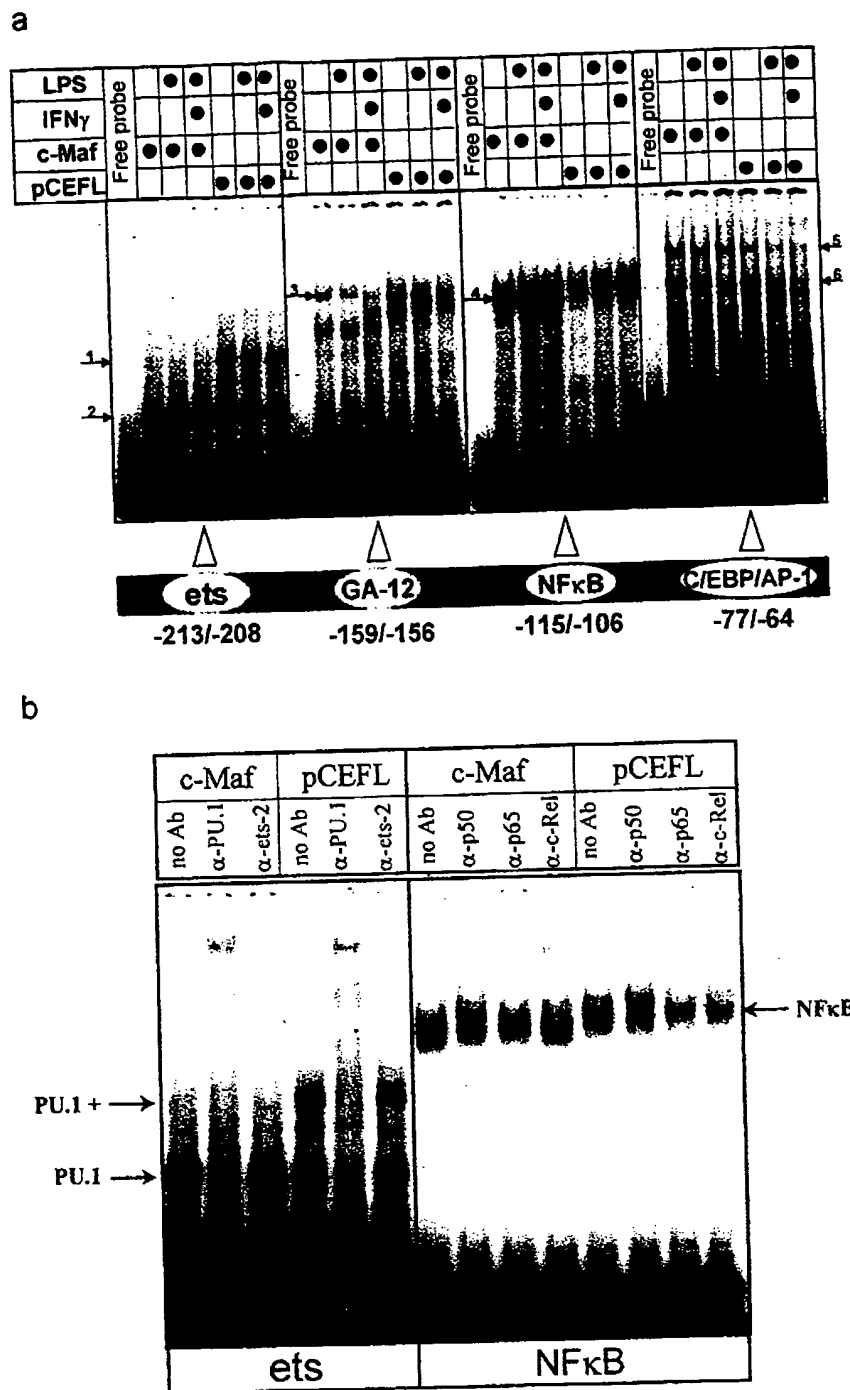
#### *IL-10* production in c-Maf-deficient macrophages is impaired, whereas *IL-10*-mediated inhibition of *IL-12 p40* production is intact

An important question was whether c-Maf is the sole mediator of *IL-10*'s inhibitory effects on *IL-12* production by macrophages. To address this issue, we obtained c-Maf-deficient murine macrophages derived from the fetal liver of day-14 embryos because of the prevalent embryonic lethality of homozygous c-Maf deficiency (7). In c-maf-deficient macrophages derived from fetal liver (one wild type, two heterozygotes, and six homozygotes), the levels of *IL-12 p40* production induced by LPS or IFN- $\gamma$  plus LPS were comparable in the three groups while *IL-10* production was impaired in c-Maf<sup>-/-</sup> macrophages (Fig. 8a). However, *IL-10* treatment of LPS- or IFN- $\gamma$ /LPS-activated macrophages strongly suppressed *IL-12 p40* production, displaying no discernible difference from the normal or heterozygous macrophages (Fig. 8b).



**FIGURE 5.** Role of NF- $\kappa$ B in c-Maf-mediated *IL-12 p40*-suppressive effects. *a*, A human *IL-12 p40* promoter-luciferase reporter construct containing 222 bp of upstream sequence (-222) (upper panel) or a mutant construct that contains base substitutions within the NF- $\kappa$ B site located at -109 (lower panel) was cotransfected transiently with a c-Maf expression vector or the control vector pCEFL into RAW264.7 cells at a molar ratio of 1:1 (reporter:effector). Cells were subsequently stimulated with LPS alone or IFN plus LPS. Cell lysates were assayed for luciferase activity. *b*, An NF- $\kappa$ B-dependent luciferase reporter construct (upper panel) or the 3.3-kb human *IL-12 p40* reporter construct (lower panel) was cotransfected transiently with c-Maf or pCEFL. The data are summaries of two independent experiments.

**FIGURE 6.** DNA-protein interactions at various sites of the *IL-12 p40* promoter. *a*, EMSA was performed to examine the DNA-binding activities at the ets, GA-12, NF- $\kappa$ B, C/EBP, and AP-1 sites on the *IL-12 p40* promoter using nuclear extracts prepared from RAW264.7 cells following transient transfection with c-Maf or the control vector pCEFL, and stimulation of the cells with LPS or IFN- $\gamma$  plus LPS. The oligonucleotide probes containing the relevant sites are indicated by  $\Delta$  together with their respective names and promoter coordinates. The various complexes are indicated by numbers. *b*, Supershift EMSA was performed to identify the components of the complexes induced under various conditions. Two nuclear extracts were used in this procedure: IFN- $\gamma$  and LPS-stimulated RAW264.7 cells with or without c-Maf expression by transfection. The identified complexes are indicated by their names.



## Discussion

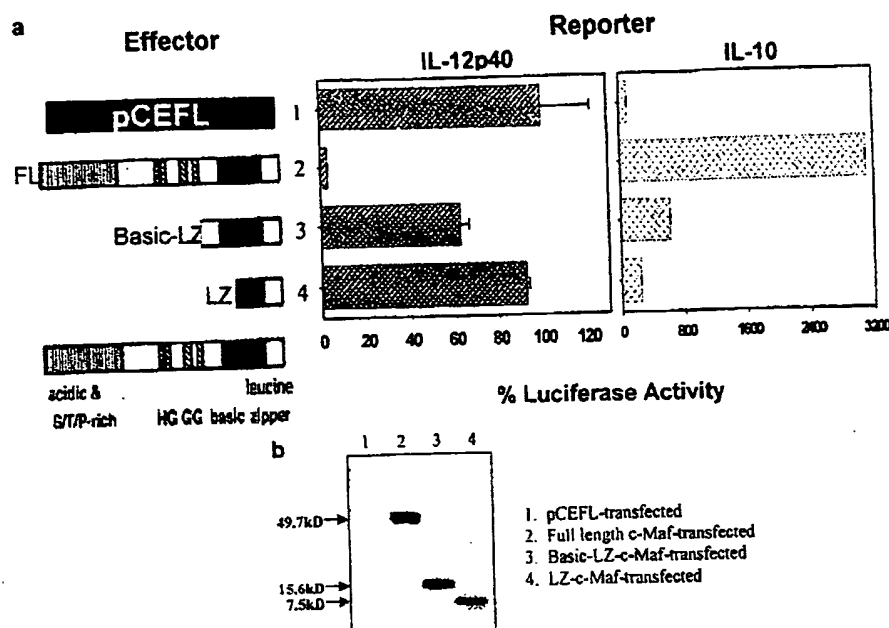
**Discussion**

In this study, we took a genome-wide approach to search for genes that are induced by IL-10 in pathogen/cytokine-activated human monocytes with a further objective to identify those that are involved in the inhibition of IL-12 production.

The observation that all of the genes identified by this approach including c-Maf are constitutively expressed and that IL-10 treatment merely reverses their inhibition by macrophage-activating agents suggests that IL-10's general function may be to maintain a homeostasis of cellular activities. In other words, the intrinsic activities of IL-10 are to bring an activation state back to a resting state in a reactionary manner, as opposed to a "proactive" function in which IL-10 would directly seek to turn on genes.

We also demonstrated for the first time that IL-10 and c-Maf are capable of enhancing each other's expression, forming a positive amplification loop that is likely to reinforce one another's activities, leading to a profound impact.

We have partially localized the c-Maf-responsive element within the *IL-12 p40* promoter by a reductionist approach of deletions and mutations to a region downstream of the C/EBP and AP-1 site (Fig. 4), but upstream of +20 (data not shown). Our AP-1 mutant did not result in any reduction in the *p40* promoter activity induced by IFN- $\gamma$  and LPS, a result that differs from that of Zhu et al. (33). The reason for this discrepancy is not immediately clear. Possibly, species differences could account for such a discrepancy in that the human *IL-12 p40* promoter-luciferase

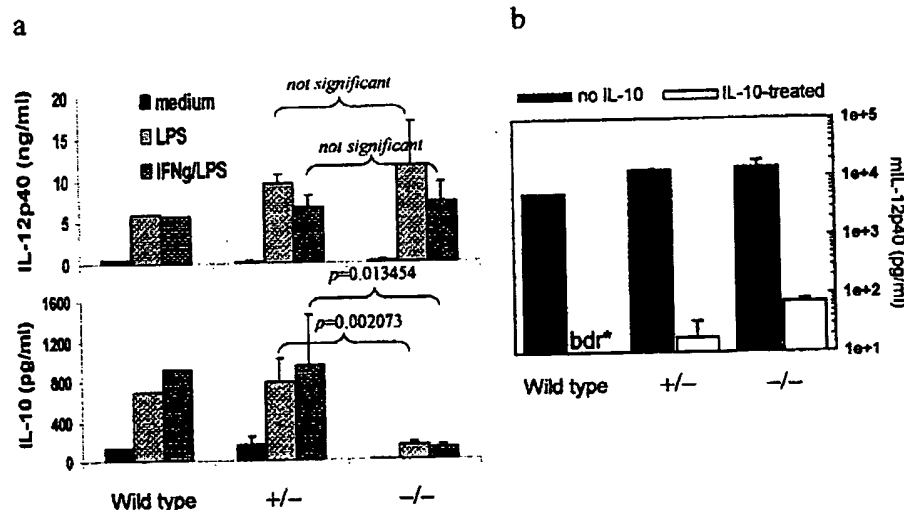


**FIGURE 7.** Inhibition of *IL-12 p40* transcription and activation of *IL-10* transcription by c-Maf requires its N-terminal transactivation domain. *a*, Transient cotransfections were performed as described in Fig. 5 using *IL-12 p40* or *IL-10*-luciferase reporters with the wild-type c-Maf expression vector or its deletion derivatives. The control was IFN- $\gamma$ /LPS- (for *IL-12 p40*) and LPS-stimulated cells (for *IL-10*), which maximize their respective promoter activation (see Fig. 3a). Luciferase activities are expressed as percentage of that of the control. FL, full-length c-Maf; basic-LZ, c-Maf construct containing no trans-activation domain but retaining the DNA-binding (basic) and dimerization (LZ) domains; LZ, c-Maf construct containing only the protein dimerization domain. Some basic structural features of the wild-type c-Maf are indicated. *b*, Nuclear protein expression of the transfected c-Maf constructs was analyzed by Western blot using a flag Ab (hemagglutinin-tag). Note that the nuclear extracts were derived from RAW264.7 cells stimulated with IFN- $\gamma$  and LPS. Data are representative of three independent experiments.

reporter was used in our study and the study in which the role of the AP-1 site was investigated used the murine *IL-12 p40* promoter linked to a CAT reporter.

Analyses of direct nuclear DNA-binding activities at the critical sites such as ets, GA-12, NF- $\kappa$ B, and C/EBP/AP-1 elements showed significant changes induced by c-Maf expression (Fig. 6).

However, these changes do not seem to be essential for c-Maf-mediated inhibition of *p40* transcription as removal of promoter regions harboring these sites or site-directed mutagenesis in some of these elements did not impact on c-Maf's ability to inhibit the *p40* promoter activity. Thus, these sites targeted by c-Maf are redundant with respect to c-Maf-mediated inhibition, and the precise



**FIGURE 8.** IL-10 production is impaired in c-Maf-deficient macrophages, whereas IL-12 p40 production and its inhibition by IL-10 are intact. Macrophages were derived from fetal liver extracted from day 14 embryos of c-Maf heterozygous (+/-) pregnant mothers; their genotypes were determined by PCR as described in *Materials and Methods*. Cells were plated at  $1 \times 10^5$ /well, stimulated with LPS or IFN- $\gamma$  plus LPS in the presence or absence of IL-10 (10 ng/ml). Twenty-four hours following stimulation, cells were harvested and cell-free supernatant assayed for mIL-12 p40 and mIL-10 production by ELISA. *a*, IL-12 p40 and IL-10 production following stimulation. Data are derived from one wild-type, two heterozygous, and six homozygous embryos with SEM. *b*, IL-10 treatment-induced inhibition of IL-12 p40 production in IFN- $\gamma$  plus LPS-stimulated macrophages. This separate experiment involved one wild-type (+/+), two heterozygous (+/-), and five homozygous (-/-) embryos. The lower limits of the ELISA were 15.6 pg/ml for mIL-12 p40 and 31 pg/ml for mIL-10. bdr, below detection range.

# REGULATION OF IL-12 AND IL-10 GENE EXPRESSION BY c-Maf

location of the essential putative c-Maf-response element remains elusive.

The requirement of the N-terminal domain of c-Maf for its IL-12-inhibiting activity (Fig. 7a) has two implications. The first is that c-Maf may act directly on the p40 promoter in conjunction with an additional factor producing a repressive outcome. This is unlikely since we were unable to identify a site on the p40 promoter to which c-Maf or its associated forms bind (data not shown). The second possibility is that the inhibitory effect of c-Maf may be mediated through an intermediary, i.e., c-Maf induces another factor which in turn suppresses IL-12 p40 transcription. A precedent of such an indirect repression mechanism by c-Maf was reported by Hegde et al. (34), who showed that expression of c-Maf in human immature myeloblastic cells inhibited the transcription of the myeloid lineage-restricted CD13/APN gene by inducing the binding of both c-Myb and ets-1 to the promoter without its own direct interaction with the target gene. In a preliminary experiment to identify this putative intermediate factor using the Affymetrix microarray containing 12,600 genes and comparing mRNA expression profiles of Ad/c-Maf-transduced human macrophages vs Ad/GFP-transduced cells, four nuclear factors were found to be significantly induced by c-Maf expression ( $\geq 2$ -fold). Interesting and relevant among these is the protein inhibitor of activated STAT protein (PIASx- $\alpha$ ), which inhibits Stat1-mediated gene activation (35). The uncovering of this transcriptional repressor in c-Maf-mediated inhibition of IL-12 gene expression is most likely related to the fact that our cells had been treated with IFN- $\gamma$ , which induces Stat1. Further investigation is in progress.

The preferential inhibition of IL-12 gene transcription by c-Maf is not surprising given the previous demonstration of its highly selective activation of IL-4 transcription without affecting the expression of many other Th2 cytokines (10). Our observation that TNF- $\alpha$  expression is not regulated by c-Maf (data not shown) suggests that other mediators of IL-10's broad anti-inflammatory activities exist that operate independently of c-Maf to modulate inflammation. Some of the other genes identified in our limited search (summarized in Table I) likely play a role in the multiple regulatory pathways controlled by IL-10. For example, the stress-inducible protein HO-1 provides protection against oxidative stress. This anti-inflammatory activity of HO-1 is mediated by carbon monoxide, a by-product of heme catabolism by HO. Furthermore, carbon monoxide mediates its anti-inflammatory effects, including the inhibition of LPS-induced expression of TNF- $\alpha$ , IL-1 $\beta$ , and macrophage-inflammatory protein-1 $\beta$  and enhancement of IL-10 production through a pathway involving the p38 MAPK (36).

Members of the Maf family of bZIP transcription factors can affect transcription in either a positive or negative fashion, depending on their particular protein partner and the context of the target promoter (14, 34, 37-45). This functional duality of c-Maf is consistent with our observation that c-Maf can switch from being a transcriptional repressor of the IL-12 p40 promoter containing sequences extending 5' beyond the NF- $\kappa$ B site at -115, to an activator of the p40 promoter which is deleted down to the NF- $\kappa$ B site or further downstream (Fig. 4). The underlying mechanism of this specific switch is not presently understood. It may involve dynamic interactions between c-Maf-induced complexes and pathogen/cytokine-activated transcription factors which bind to sites upstream of the NF- $\kappa$ B element in the context of preassembled basal transcription machinery.

The apparent dispensability of c-Maf in IL-10-mediated inhibition of IL-12 production in fetal liver-generated macrophages (Fig. 8b) indicates a functional redundancy in this pathway. We have preliminary evidence indicating that endogenous AP-1 is a transcriptional inhibitor of the IL-12 p40 gene, and IL-10's inhibition

of IL-12 p40 protein synthesis is dependent, at least in part, on AP-1 (our published data), suggesting that AP-1 could be such an alternative mediator. In support of this supposition, it is noted that p38 MAPK, an essential activator of AP-1, is induced by IL-10 in activated human macrophages (Table I). In ovarian and endometrial carcinoma cells, IL-10 directly stimulates AP-1 activity (46).

Alternatively, the IL-10 dependency of c-Maf for its transcriptional repression of IL-12 p40 may be different between mouse and human, or between liver macrophages and those from other anatomical compartments such as spleen and peritoneal cavity. Presently, technical difficulties preclude direct demonstration and discerning of these possibilities.

In summary, we have established a novel role of c-Maf in the selective and opposing regulation of IL-10 and IL-12 gene transcription in macrophages. Yet, c-Maf is apparently a redundant mediator of IL-10's suppressive activity on IL-12 production. The implications are 2-fold. It suggests the possibility that c-Maf, as an immunological regulator, could play a dual role of driving humoral immunity via stimulation of IL-4 transcription, and suppressing innate immune responses by inhibiting IL-12 production. It also implies that the other property of c-Maf, its oncogenic potential, could be exerted through its intimate interaction with the immune surveillance of the host and manifested in the form of selective inhibition of such critical activators of tumor-busting CTL and NK cells as IL-12.

## References

- Trinchieri, G. 1998. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv. Immunol.* 70:83.
- Trinchieri, G., and P. Scott. 1999. Interleukin-12: basic principles and clinical applications. *Curr. Top. Microbiol. Immunol.* 238:57.
- Aste-Amezaga, M., X. Ma, A. Sartori, and G. Trinchieri. 1998. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *J. Immunol.* 160: 5936.
- Blank, V., and N. C. Andrews. 1997. The Maf transcription factors: regulators of differentiation. *Trends Biochem. Sci.* 22:437.
- Chesi, M., P. L. Bergsagel, O. O. Shonukan, M. L. Martelli, L. A. Brents, T. Chen, E. Schrock, T. Ried, and W. M. Kuehl. 1998. Frequent dysregulation of the c-maf proto-oncogene at 16q23 by translocation to an Ig locus in multiple myeloma. *Blood* 91:4457.
- Chesi, M., W. M. Kuehl, and P. L. Bergsagel. 2000. Recurrent immunoglobulin gene translocations identify distinct molecular subtypes of myeloma. *Ann. Oncol.* 11:131.
- Kim, J. I., T. Li, I. C. Ho, M. J. Grusby, and L. H. Glimcher. 1999. Requirement for the c-Maf transcription factor in crystallin gene regulation and lens development. *Proc. Natl. Acad. Sci. USA* 96:3781.
- Kawachi, S., S. Takahashi, O. Nakajima, H. Ogino, M. Morita, M. Nishizawa, K. Yasuda, and M. Yamamoto. 1999. Regulation of lens fiber cell differentiation by transcription factor c-Maf. *J. Biol. Chem.* 274:19254.
- Ring, B. Z., S. P. Cordes, P. A. Overbeck, and G. S. Barsh. 2000. Regulation of mouse lens fiber cell development and differentiation by the Maf gene. *Development* 127:307.
- Kim, J. I., I. C. Ho, M. J. Grusby, and L. H. Glimcher. 1999. The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. *Immunity* 10:745.
- Ma, X., J. M. Chow, G. Gri, G. Carra, F. Gerosa, S. F. Wolf, R. Dzilo, and G. Trinchieri. 1996. The interleukin 12 p40 gene promoter is primed by interferon  $\gamma$  in monocytic cells. *J. Exp. Med.* 183:147.
- Gri, G., D. Savio, G. Trinchieri, and X. Ma. 1998. Synergistic regulation of the human interleukin-12 p40 promoter by NF- $\kappa$ B and Ets transcription factors in Epstein-Barr virus-transformed B cells and macrophages. *J. Biol. Chem.* 273: 6431.
- Grazia Cappiello, M., F. S. Sutterwala, G. Trinchieri, D. M. Mosser, and X. Ma. 2001. Suppression of IL-12 transcription in macrophages following Fc $\gamma$  receptor 3 is an oncogene that contributes to tumor progression in multiple myeloma. *Blood* 97:729.
- Cepko, C. 2001. Preparation of a specific retrovirus producer cell line. In *Current Protocols in Molecular Biology*, Vol. 2. F. M. Ausubel, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, K. Struhl, eds. Wiley, p. 9.10.9.

17. Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with "mini-extracts", prepared from a small number of cells. *Nucleic Acids Res.* 17:6419.
18. Ma, X., M. Neurath, G. Gri, and G. Trinchieri. 1997. Identification and characterization of a novel Ets-2-related nuclear complex implicated in the activation of the human interleukin-12 p40 gene promoter. *J. Biol. Chem.* 272:10389.
19. Lichanska, A. M., and D. A. Hume. 2000. Origins and functions of phagocytes in the embryo. *Exp. Hematol.* 28:601.
20. D'Amico, G., G. Frascaroli, G. Bianchi, P. Transidico, A. Doni, A. Vecchi, S. Sozzani, P. Allavena, and A. Mantovani. 2000. Uncoupling of inflammatory chemokine receptors by IL-10: generation of functional decoys. *Nat. Immunol.* 1:387.
21. Nakamura, H., J. Vaage, G. Valen, C. A. Padilla, M. Bjornstedt, and A. Holmgren. 1998. Measurements of plasma glutaredoxin and thioredoxin in healthy volunteers and during open-heart surgery. *Free Radic. Biol. Med.* 24:1176.
22. Nath, K. A., G. M. Vercellotti, J. P. Grande, H. Miyoshi, C. V. Paya, J. C. Manivel, J. J. Haggard, A. J. Croatt, W. D. Payne, and J. Alam. 2001. Heme protein-induced chronic renal inflammation: suppressive effect of induced heme oxygenase-1. *Kidney Int.* 59:106.
23. Rauschmayr, T., R. W. Groves, and T. S. Kupper. 1997. Keratinocyte expression of the type 2 interleukin 1 receptor mediates local and specific inhibition of interleukin 1-mediated inflammation. *Proc. Natl. Acad. Sci. USA* 94:5814.
24. Behrens, A., K. Sabapathy, I. Graef, M. Cleary, G. R. Crabtree, and E. F. Wagner. 2001. Jun N-terminal kinase 2 modulates thymocyte apoptosis and T cell activation through c-Jun and nuclear factor of activated T cell (NF-AT). *Proc. Natl. Acad. Sci. USA* 98:1769.
25. Chen, N., M. Nomura, Q. B. She, W. Y. Ma, A. M. Bode, L. Wang, R. A. Flavell, and Z. Dong. 2001. Suppression of skin tumorigenesis in c-Jun NH(2)-terminal kinase-2-deficient mice. *Cancer Res.* 61:3908.
26. Song, G. Y., C. S. Chung, D. Jarrar, I. H. Chaudry, and A. Ayala. 2001. Evolution of an immune suppressive macrophage phenotype as a product of P38 MAPK activation in polymicrobial sepsis. *Shock* 15:42.
27. Wang, I. M., C. Contursi, A. Masumi, X. Ma, G. Trinchieri, and K. Ozato. 2000. An IFN- $\gamma$ -inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. *J. Immunol.* 165:271.
28. Murphy, T. L., M. G. Cleveland, P. Kulesza, J. Magram, and K. M. Murphy. 1995. Regulation of interleukin 12 p40 expression through an NF- $\kappa$ B half-site. *Mol. Cell. Biol.* 15:5258.
29. Sanjabi, S., A. Hoffmann, H. C. Liou, D. Baltimore, and S. T. Smale. 2000. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc. Natl. Acad. Sci. USA* 97:12705.
30. Weinmann, A. S., D. M. Mitchell, S. Sanjabi, M. N. Bradley, A. Hoffmann, H. C. Liou, and S. T. Smale. 2001. Nucleosome remodeling at the IL-12 p40 promoter is a TLR-dependent, Rel-independent event. *Nat. Immunol.* 2:51.
31. Plevy, S. E., J. H. Gemberling, S. Hsu, A. J. Dorner, and S. T. Smale. 1997. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol. Cell. Biol.* 17:4572.
32. Becker, C., S. Wirtz, X. Ma, M. Blessing, P. R. Galle, and M. F. Neurath. 2001. Regulation of IL-12 p40 promoter activity in primary human monocytes: roles of NF- $\kappa$ B, CCAAT/enhancer-binding protein  $\beta$ , and PU.1 and identification of a novel repressor element (GA-12) that responds to IL-4 and prostaglandin E(2). *J. Immunol.* 167:2608.
33. Zhu, C., K. Gagnidze, J. H. Gemberling, and S. E. Plevy. 2001. Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter: demonstration of novel functional elements by a reductionist approach. *J. Biol. Chem.* 276:18519.
34. Hegde, S. P., A. Kumar, C. Kurschner, and L. H. Shapiro. 1998. c-Maf interacts with c-Myb to regulate transcription of an early myeloid gene during differentiation. *Mol. Cell. Biol.* 18:2729.
35. Liu, B., J. Liao, X. Rao, S. A. Kushner, C. D. Chung, D. D. Chang, and K. Shuai. 1998. Inhibition of Stat1-mediated gene activation by PIAS1. *Proc. Natl. Acad. Sci. USA* 95:10626.
36. Omerbein, L. E., F. H. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R. J. Davis, R. A. Flavell, and A. M. Choi. 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat. Med.* 6:422.
37. Igarashi, K., K. Kataoka, K. Itoh, N. Hayashi, M. Nishizawa, and M. Yamamoto. 1994. Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. *Nature* 367:568.
38. Kataoka, K., K. T. Fujiwara, M. Noda, and M. Nishizawa. 1994. MafB, a new Maf family transcription activator that can associate with Maf and Fos but not with Jun. *Mol. Cell. Biol.* 14:7581.
39. Kataoka, K., M. Noda, and M. Nishizawa. 1994. Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. *Mol. Cell. Biol.* 14:700.
40. Kerppola, T. K., and T. Curran. 1994. Maf and Nrl can bind to AP-1 sites and form heterodimers with Fos and Jun. *Oncogene* 9:675.
41. Kerppola, T. K., and T. Curran. 1994. A conserved region adjacent to the basic domain is required for recognition of an extended DNA binding site by Maf/Nrl family proteins. *Oncogene* 9:3149.
42. Kataoka, K., K. Igarashi, K. Itoh, K. T. Fujiwara, M. Noda, M. Yamamoto, and M. Nishizawa. 1995. Small Maf proteins heterodimerize with Fos and may act as competitive repressors of the NF-E2 transcription factor. *Mol. Cell. Biol.* 15:2180.
43. Kurschner, C., and J. I. Morgan. 1995. The maf proto-oncogene stimulates transcription from multiple sites in a promoter that directs Purkinje neuron-specific gene expression. *Mol. Cell. Biol.* 15:246.
44. Kataoka, K., M. Noda, and M. Nishizawa. 1996. Transactivation activity of Maf nuclear oncoprotein is modulated by Jun, Fos and small Maf proteins. *Oncogene* 12:53.
45. Sieweke, M. H., H. Tekotte, J. Frampton, and T. Graf. 1996. MafB is an interaction partner and repressor of Ets-1 that inhibits erythroid differentiation. *Cell* 85:49.
46. Seppanen, M., T. Henttinen, L. Lin, J. Punnonen, S. Grenman, R. Punnonen, and K. K. Vihko. 1998. Inhibitory effects of cytokines on ovarian and endometrial carcinoma cells in vitro with special reference to induction of specific transcriptional regulators. *Oncol. Res.* 10:575.